

nature

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See how they run

THE chart in the centre of this week's issue of *Nature* is not, you will be disappointed to hear, the result of furtive investigations by the staff under trying conditions. None of our sub-editors was dispatched with binoculars to phone-boxes in Whitehall to catch glimpses of faceless men doing a quick sprint out of their ministry to black government cars waiting to take them to assignments in other ministries. No laser beams were trained on the curtained windows behind which whispered conferences were being held. All the information is in the public domain. The research was no more exciting than several days of delving through annual reports, some telephone calls and a few fivopenny bus rides to pick up official documents. And in a way the product of the research isn't exciting either. Old hands on the London scene will look at the chart and say that there is nothing on it they didn't know about already—and that there is enough inter- and intra-departmental committee structure in Whitehall to convert our simple little picture into a veritable cat's cradle of inter-connections.

Indeed the old hands are right. The proliferation of working parties, study groups, panels and sub-committees is unending and we could have easily devoted six months of our time to trying to put together a coherent picture of this fine detail. But how many are there who can even be called old hands? The majority of British scientists will probably find that even our elementary chart reveals quite a lot they hadn't realised before. Who runs Kew? Harwell? What ministry pays for space research? How much is spent on defence? How are we represented in Europe? At UNESCO?

It is, of course, almost in the very nature of a central bureaucracy almost exclusively concentrated in one city,

that those on the outside will take a Kafka-esque view of it. Contact with the outside world tends to be through the trusted advisers or council members; criticism from the press is greeted with stony silence; communications from outside tend to leave the individual feeling impotent. And yet the castle is fairly easily penetrable to those who take along a map, and most of its influential inhabitants have been scientists themselves. Moreover, because of the perennial problem that central bureaucracy has in accurately judging the feelings of those at the grass roots, anyone with the ability to communicate with the right bureaucrat at the right time can have a disproportionate influence—for good or bad.

Hardly anyone on this chart could be said to have got there by anything resembling a democratic process, and by the same token hardly anyone could be removed by anything resembling a democratic process. We are not about to advocate annual elections to councils and committees; what we do urge, however, is a much wider understanding of the rudiments of science policy-making, and maybe this chart will help.

Only one committee which we wished to list has had to remain ex-directory. The Defence Scientific Advisory Committee, we have been told (not by its chairman), prefers to remain anonymous, partly for fear of student action against its members. In view of the immense expenditure on defence research and the almost complete ignorance in the academic and student world of defence matters, anonymity can only serve to create further alienation.

● Extra copies of the chart are available. See page iii for details.

Earthquakes cause cancer: official probe shock

THE annual British Association meeting is upon us again, and so is the silly season for newspapers. Here are a few simplistic headlines science could well do without in the coming week.

Coffee-drinking causes cancer, scientist says

British astronomy leads the world

Concorde's effects harmless—government scientist

Cancer drug hopes

Sex at 80 keeps you fit—scientist

Earthquakes threaten North Sea oil

Tea-drinking causes cancer

Concorde 'will end world as we know it'—environmentalist

British astronomy in doldrums

New X-ray star no hazard to health

Scientists create life in laboratory

Can readers of *Nature* think of any others?



Weather watchers

As fewer and fewer agricultural regions have supplied increasing amounts of the food consumed by the countries where population growth is greatest, there has been growing concern about how the climate of the next few decades may affect world agricultural productivity. Henry Lansford, of the National Center for Atmospheric Research, Boulder, Colorado, reports.

SOME atmospheric scientists, projecting trends of the past 30 years or so, foresee steadily decreasing temperatures accompanied by greater climatic variability—more droughts, floods, unseasonable cold spells, and other extreme events that certainly would reduce agricultural productivity, at least in the regions where they occurred. Professor Hubert Lamb, of the University of East Anglia, and Professor Hermann Flohn, of the University of Bonn have warned that this sort of climatic change seems to be in progress, together with Dr Reid Bryson, of the University of Wisconsin, and Dr Walter Orr Roberts, who formerly headed the National Center for Atmospheric Research (NCAR) and is now with the Aspen Institute for Humanistic Studies.

Other climatologists maintain that the climatic trend of recent years does not necessarily represent a long term change, but should be viewed as a fluctuation that may halt or reverse itself at any time. These climatological conservatives maintain that our understanding of the mechanisms of climatic change is so rudimentary that no sound basis exists for assuming that a downward trend in temperature will continue in the future. Among these conservatives are Dr B. J. Mason, who heads the British Meteorological Office, and people like Dr J. Murray Mitchell of the National Oceanic and Atmospheric Administration (NOAA), and Professor Helmut Landsberg, of the University of Maryland.

But even these conservatives are worried about the impact of climatic variability on world food supplies. The potential consequences of droughts and other climatic anomalies are greater than ever before because of present low levels of grain reserves, increasing use of semi-arid and other marginal lands for farming, and expectations of continuing high yields

from the new "Green Revolution" crops that are highly responsive to generous supplies of water and fertiliser.

During the past year and a half, atmospheric and agricultural scientists, as well as specialists in fields such as economics, international development, law and political science, have gathered for a succession of international conferences designed to identify some of the major problems of climate-food-social interactions and to try to develop new research strategies for attacking those problems effectively. The meetings have included:

- A conference on "Weather and Climate Change, Food Production and Interstate Conflict" held in New York City by the Rockefeller Foundation in January 1974
- A workshop on "The Impact on Man of Climate Change" held at the University of Bonn by the International Federation of Institutes for Advanced Study (IFIAS) in May 1974
- A conference on "World Food Supply in Changing Climate" held at Sterling Forest, New York, in December 1974 under the joint sponsorship of the American Society of Agronomy and the Aspen Institute for Humanistic Studies
- A pair of workshops, one on the policy implications of food and climate interactions and the other on the design of a study of the social, political, economic and ethical impacts of drought, held in West Berlin by IFIAS and the Aspen Institute in February 1975
- A conference on "Climate Change, Food Production and Interstate Conflict" held by the Rockefeller Foundation at Bellagio, Italy, in June 1975.

Although the participants at these meetings disagreed about a great many things, there seems to be fairly general agreement that the vagaries of climate do indeed represent a serious threat to world food supplies in

these precarious times of rapidly growing demand for food, low world grain reserves and increasing dependence of many heavily populated nations on a few regions of high agricultural productivity, usually halfway around the world from the regions of high consumption, for their food supplies.

The conclusions, recommendations, and resolutions that have come out of these conferences have ranged from the strident to the cautious. The IFIAS statement that emerged from the Bonn meeting, for example, said that:

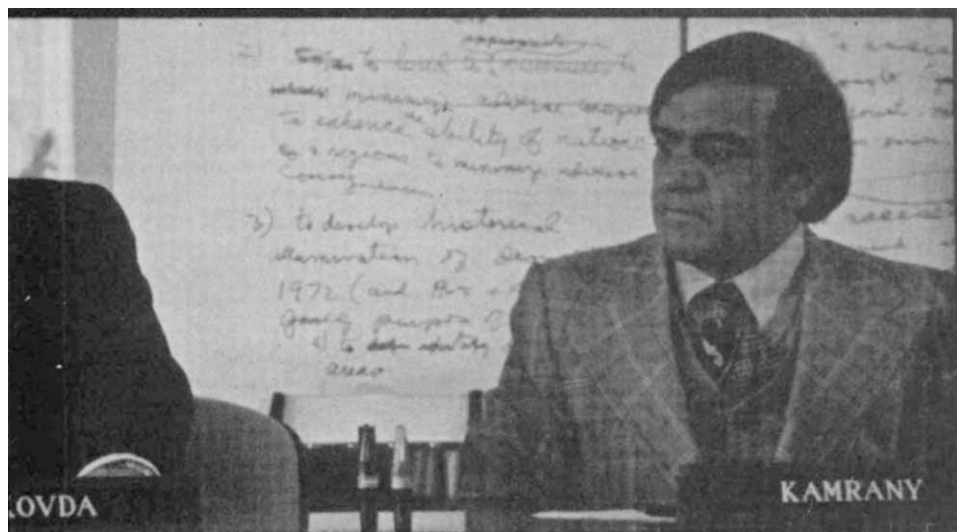
"The nature of climatic change is such that even the most optimistic experts assign a substantial probability of major crop failures within a decade. If national and international policies do not take such failures into account, they may result in mass deaths by starvation and perhaps in anarchy and violence that could exact a still more terrible toll."

With more reserve and less rhetoric, the participants in the recent Bellagio Conference came to much the same conclusion. They agreed that:

"... there is some cause to believe—although it is far from certain—that climatic variability in the remaining years of this century may be even greater than during the 1940–70 period", and concluded that "greater climatic variability than that of the last two decades could cause major crop failures quite beyond the current capability of agricultural science and technology to control or mitigate."

Although these climate-food meetings have not resulted in any solid consensus among atmospheric and agricultural scientists about just what the climate is going to do and how it will affect food supplies, much less about what international policies and mechanisms will be needed to cope effectively with conflicts that may develop among nations as a result, a few common conclusions seem to be





Walter Roberts, Nake Kamrany and Viktor Korda in Berlin.

emerging from all the discussion and debate.

As far as the climate itself is concerned, there is fairly general agreement on two points:

- The climate of the first half of the twentieth century was considerably warmer than the average of the past 1,000 years, which suggests the possibility that cooler weather can be expected to follow.

- The average global temperature, which gradually increased during the first half of the century, has been decreasing during the past quarter of a century, at least at latitudes above 55°N. This cooling was between 2 and 3 °C for some northern locations such as Iceland. Lamb points out, however, that the length of the growing season in England increased by two to three weeks during the first half of the century and has dropped back by about two weeks since 1945.

There is considerable disagreement about whether or not the cooling trend is likely to continue. One school of opinion, which includes Bryson, Flohn, Lamb and Roberts, sees a strong probability that the downturn in temperature that has occurred since 1950 will continue for at least another two or three decades. Other climatologists such as Mitchell and Landsberg maintain that the causes and mechanisms of climatic change are not understood well enough for anyone to make such a prediction with any assurance of success.

Although a number of the scientists who anticipate a global downturn in temperature participated in the Bellagio Conference, the group as a whole took a cautious view of the question of global cooling. The conference participants concluded that:

"It is not certain that the decline in Northern Hemisphere temperature will continue; it may halt or reverse itself. Even if the global average temperature were to decrease steadily over the next

20 years, it is unlikely that it would drop as much as one degree Celsius."

But they went on to point out that: "temperature change *per se* is not the most serious climatic threat to food production. The possibility of increased climatic variability, particularly in distribution of precipitation, is of greater concern."

The question of variability is another subject of controversy. Bryson and others believe that the cooling trend in the high latitudes of the Northern Hemisphere has changed large scale atmospheric circulation patterns by increasing the temperature contrast between tropical and polar regions. They believe that this has produced increased climatic variability—a higher incidence of droughts, floods, unseasonable cold weather and other extreme events that have characterised recent bad crop years such as 1972 and 1974.

Whether or not the climate is growing more variable, even the climatological conservatives such as Landsberg agree that climatic variability represents a continuing threat to uniform crop yields in many agricultural regions. Although the Bellagio Conference participants did not conclude that the climate is becoming increasingly variable, they agreed that "climatic variability—region by region and from year to year in particular regions—is and will continue to be large."

In discussing problems of climate and food, atmospheric scientists have frequently referred to the climate of the first half of this century as "benign," implying, perhaps unintentionally, that it provided the best possible growing conditions for crops everywhere. But at Bellagio the agricultural scientists pointed out that this is not so. The frequency of droughts in the Soviet Union increased sharply during that period. In parts of the mid-western United States, summer temperatures have often been too hot for

optimum yields from some crops that are grown there. A general cooling trend, while shortening the growing season in some northern regions, might result in a compensating increase in crop yields in regions further south. Professor Lamb has suggested that, in discussions of climate change, it would be wise to avoid using the words "benign" and "normal," as they both imply value judgments that are subject to considerable argument.

After listing some of the impacts that a cooling trend might have on agriculture—changes in distribution of precipitation, shorter growing seasons in high latitudes, and cooler growing temperatures in some regions—the Bellagio participants admitted that:

"Some effects of such climatic changes would be beneficial to agriculture and others would be detrimental, but the net effect, for the whole world or for any particular region, is not known."

To remedy this lack of knowledge of the agricultural impact of future climatic developments, it was recommended that a set of 'scenarios' specifying plausible future climatic sequences be developed and that the impact of each possible climatic future on agricultural production, the world food situation and international relations be assessed as precisely and quantitatively as possible.

Regardless of the changing casts of characters, different approaches, and specific conclusions that have characterised the recent climate-food meetings, one grim theme emerges from all of them. Whether or not any long term change to cooler temperatures or increasing climatic variability is in progress, there is a high probability that major regional and world food emergencies will occur during the next decade or two as a result of serious reductions in crop yields or major crop failures caused by climatic anomalies.

A number of possible research and policy strategies have emerged that could contribute to effective national and international responses to such emergencies.

It seems that the atmospheric scientists should accelerate their efforts to determine whether or not it is possible to predict climatic conditions a season or more in advance, as well as examining ways in which existing climatic information can be made more useful in coping with agricultural problems. An improved understanding of the workings and interactions of the five physical elements of the total climatic system—atmosphere, hydrosphere, cryosphere, lithosphere and biosphere—is badly needed. Both theoretical and empirical approaches to problems of climate change should be pursued. Agricultural scientists should

assess the potential impact of various climatic anomalies on various crops, particularly the new high yield varieties, and should consider plant breeding strategies, as well as techniques of crop, soil and water management, that can reduce the impact of climatic stresses on agricultural productivity. Special attention should be given to the role of soils in mitigating the impacts of drought, and to the improvement of dryland-farming practices, as more and more semi-arid lands are brought under cultivation.

Application of new and existing agricultural technology to improvement of crop yields in regions that supply food for their own consumption seems to be at least as valuable in the long run as striving to continue to increase yields in exporting regions such as the United States and Canada.

Policy makers must assess national and international policies on agriculture and food in the light of the probability that major food shortages,

affecting large regions if not the entire world, can be expected to result from climatic stresses over the next few decades. Although years of bad climate such as 1972 may not necessarily reduce total world food production, they clearly have the potential for disrupting world food trade patterns and raising prices to the extent that people in some regions will go hungry not because there is no food, but because it costs so much that they cannot afford to eat properly. The potential is great for conflict among nations as well as for human suffering in the regions that are affected directly by climatic anomalies.

There does not seem to be any miraculous 'technological fix' waiting in the wings to save the day. Agricultural technology probably can be applied to achieve additional increases of 100% or more in crop yields in some regions, primarily those where food consumption is highest, but such increases are not likely in most of the present major food-producing regions.

Climate forecasting will be useful only if workable alternatives are available so that the farmer can respond to the foreknowledge that a drought or a short growing season is imminent. Weather-modification technology may have the potential for improving crop yields in some regions under some conditions, but it has not been demonstrated that cloud seeding can do much, if anything, to remedy major climatic anomalies such as large scale droughts.

Science and technology certainly have appreciable contributions to make to solutions to our growing climate-food-society problems. But the evidence that has emerged from the recent conferences indicates clearly that the responsibility for establishing a framework within which scientific and technological knowledge can be applied effectively to solving the problems rests as much with the policy makers as it does with the plant breeders, the climatologists and the cloud seeders. □

A small experiment now taking place in Britain seems to be having results that warrant a little more attention from our drought-stricken sociological experts. I refer to the resistance of the Anglo-Saxon mind to metrication, a resistance which, whether inwardly sullen or sudden, is outwardly passive so long as our feet are not pushed to the wall. For the inevitably vociferous, the issue is seen in the emotive terms of an unimpeachable heritage threatened from without, and the City Editor of the *Sunday Telegraph* euphorically praises "the glorious indifference of the British people [to metrication]", himself excepted, presumably.

Because, here in the USA, metric measures are now unofficially and surreptitiously appearing in all sorts of odd corners, from the food and building industries to sports events, not to mention our completely ravished science, we are not indifferent to watching the unenthusiastic attitude of the British to losing their 2,000-year-old Roman measures. Admittedly, the decimal Roman measures became a little unmetrified after the UDI of Hengist and Horsa in a petrified British culture, but Anglo-Saxon we have staunchly remained for the 1,500 years since.

Are we now to throw over that measured tradition? Galileo learned, to his cost, the strength of tradition when, in the naive cause of truth, he tried fervently to upend it overnight. Pope Gregory's reform of the calendar was regarded by the English as even more of an occasion for cheating than the recent decimalisation of £.s.d. Tradition! Should we resist and drop

metrication, except for odd corners of science that can easily be swept clean again; should we denigrate, admire or diplomatically ignore the resolution of South Africa in enforcing it by law in one fell swoop; or do we characteristically muddle on with a facade of creeping metrication that is merely a

Failing to take the point

P. A. MOHR

measure of creeping paralysis of will?

Certainly, the British Metrication Board, in urging the people of Britain to think metric for reasons of logic and utility alone, has completely misjudged the Anglo-Saxon mind. Those who oppose metrication see the ultimate argument quite clearly: metrication is alienation. Logic and possible future utility make no impact on the gloriously indifferent.

Can the Imperialists . . . (no, that won't do) . . . Can the opponents of metrication discover that the non-Anglo-Saxon world, by discarding multifarious old measuring systems, has impaired its heritage? It might make for an interesting discussion; but surely many would prefer to regard tradition as something not merely inherited, but to be fashioned for those who will inherit it, hopefully improved, from the living. Otherwise we would still be sticking to the measuring

system of Boadicea, without the benefit of those delightfully duodecimal Roman "uncias" of length and weight. (British readers, now perhaps only able to afford their tobacco by the gram, may be interested to know that in the USA "one pound" of pipe tobacco has shrunk to 12 ounces, in precise conformity with the original definition, but not, alas, out of any respect for tradition.)

Need we go metric? Cannot measuring systems of the world co-exist as do languages? Is there a good case for Britain and the USA to drop, or stop, the whole costly and unwanted experiment and re-trench? As far as I am aware, the token metrication of Britain has barely entered everyday thought, estimation and judgment. One does not see Mr. Wilson, one of Britain's better known pragmatists, contemplating his bowl in centimetres and °C.

If metrication, an abomination of a word redolent of graduation in dietetics, really is desirable for us Anglo-Saxons, then it will not stem from the will of the people in their present mood. Either those in authority must coerce, or those favouring the change must be seen to step aside from the appointed bureaucrats of Whitehall and Washington, and start cutting ice, or, for those who disdain the methods of Sam Adams and the founders of the American Revolution, advertise the fact in the manner of the huge, state sign on the main Boston-Montreal highway: "Barre, 100 kilometers".

Maybe we can continue along the broad way, rather than take the narrow road of decision. But I fear that, in nautical parlance, we're tying ourselves in unfathomable knots.

international news

ALTHOUGH a crash research and development programme on solar energy has long been advocated by a small band of scientists and environmentalists, who see the Sun as a bountiful source of clean energy—and also by some Congressmen who see it as a useful source of votes—until recently the federal government has not seemed to share the enthusiasm. But there are now signs that the Energy Research and Development Administration (ERDA) has recast the priority of the solar energy programme it inherited from other government agencies earlier this year. Officials of ERDA are now publicly predicting that solar power could provide a very large contribution to energy needs in the United States in the twenty-first century.

The latest piece of evidence to that effect is contained in a report, published by ERDA earlier this month, in which the agency outlines an ambitious, 10-year research and development effort designed to reduce the costs of solar technologies and, it is hoped, to pave the way for their commercial introduction. ERDA states in the report that if the effort is successful, energy derived from the Sun could supply as much as 25% of the country's energy requirements by the year 2020.

Although it should be noted that the report is conspicuously devoid of cost figures—a factor which makes it difficult to compare ERDA's new proposals with previous government plans—solar enthusiasts are generally pleased that ERDA is slowly moving closer to their own predictions of what is possible (although it still has a long way to go). The new report, moreover, follows hard on the heels of the publication by

ERDA looks to the Sun

by Colin Norman, Washington

ERDA of an overall plan for energy research and development in which solar power is raised to the same level of priority as the breeder reactor and the thermonuclear power programme.

In spite of the generally optimistic tone of the report, however, ERDA is quick to caution that the potential contribution from the Sun will only be realised "if the costs of collecting and utilising solar energy can be reduced substantially"—in some cases by a factor of 100 or more.

The general approach outlined in the report is to press ahead as swiftly as practicable with demonstration projects, and to get early industrial participation in the effort to help pave the way to commercial introduction of the technology. A focal point for the programme will be a new solar energy research institute, a large laboratory which ERDA is planning to establish early next year which is expected to have a budget of about \$50 million by 1980.

The research programme is divided into three chief parts—direct thermal applications, such as solar heating and cooling; conversion of solar energy to electricity; and the development of fuels from plants.

Efforts in the first category are probably the most advanced. According to

a directive from Congress, ERDA plans to install up to 4,000 solar heating units in private homes and public buildings as part of a demonstration programme. (There are, however, unconfirmed rumours in ERDA and in Congress that the Office of Management and Budget—a powerful White House office similar in some respects to the British Treasury—will allow ERDA to install only 350 units.) The emphasis will shift to development of combined heating and cooling units in the late 1970s.

As for the conversion of solar energy to electricity, technologies to use wind power are closest to the demonstration phase. The National Aeronautics and Space Administration Agency is now building a small generating plant, and ERDA hopes to begin work next year on a larger unit. The commercial use of solar energy to generate electricity through photovoltaic cells will require some research breakthroughs to reduce the costs by a factor of at least 100, ERDA reckons, but demonstration plants with a capacity of between 1 and 5 MW are being planned for the mid-1980s. Similar sized plants for generating electricity from solar-produced steam are also planned for the mid-1980s, and in that case ERDA reckons that a cost reduction of between 50 and 70% will be required. The least advanced solar-electric concept at present is the idea of using ocean thermal gradients.

Finally, ERDA hopes to initiate a number of studies with industry in the late 1970s designed to explore the large scale use of plants and animal wastes to produce fuels such as alcohol and methane. □

New legislation on the conservation and use of mineral deposits was recently passed by the Supreme Soviet of the USSR and is to come into force on January 1, 1976.

The measures proposed do not, at first glance, seem particularly spectacular, and are concerned mainly with rational planning to reduce wastage, the introduction of new and more effective methods of ore dressing and petroleum processing, and improved methods of quality control, including the use of radioisotopes in both ferrous and non-ferrous metallurgy. The need for further prospecting for new beds and deposits and the improvement of prospecting methods is urged; this,

Legislation on use of Soviet minerals

from Vera Rich

incidentally, is regularly cited as a justification for the expenditure on the Soviet space programme, since, officially, Kosmos reconnaissance satellites are engaged, *inter alia*, in surveying mineral resources.

It is not, however, the measures themselves which attract attention so much as the urgency with which they are expressed. The Supreme Soviet "resolved: to consider as one of the

most important state problems the ensuring of rational, complex and economic use of mineral wealth and the intensification of its conservation within the aims of the further development of the socialist economy". In his address to the convocation, N. A. Tikhovov, Deputy Chairman of the Council of Ministers of the USSR, stated that in the past four years the Soviet national income has risen by 26%, the output of industry by more than 1.3 times, and real earnings by 19%. In order to continue thus to construct "the material and technical bases of a communist society", the rational use of mineral resources must be treated as a matter of great importance. Soviet

economic data are, traditionally, always presented in terms of percentages, and it is frequently difficult to refer them back to a base date, so as to calculate their meaning in real terms. Such figures for production as are readily available, however, give for the first half of 1975 240 million tons of oil, 141,000 million cubic metres of gas, 348 million tons of coal, 69.8 million tons of steel and 44 million tons of mineral fertilisers. Against this back-

ground of rapid expansion, the new laws seem to show a growing awareness that the mineral resources of the Soviet Union although vast, are still finite.

A *Pravda* editorial, dealing with the laws, stated: "Science is faced with great problems. Scientists are charged with creating new methods, techniques and technology which will allow mineral resources to be extracted without loss, with introducing them more

fully into the national economy, and with ensuring the safety of mining operations". The Academy of Sciences of the USSR, the Academies of the Union Republics, the State Committee on Science and Technology and the various scientific research organisations as well as the Ministries concerned, will all be involved in implementing the new laws.

Meanwhile, surveying for new deposits continues. □

THE Baltic countries' scientific cooperation continues. Under the auspices of the Helsinki Convention for the prevention of pollution in the Baltic Sea, a meeting was recently held in the Swedish archipelago at which Swedish and Russian biologists studied each other's methods of intercalibration of measurements in marine biological experiments. The meeting was the second in a series (the first, last year, concentrated on chemical methods of measurement) which will continue next year with the additional participation of the other signatories of the Convention: Finland, Poland, the German Democratic Republic, the Federal Republic of Germany and Denmark.

● A new input into the Swedish nuclear energy debate promises to heat the simmering controversy yet again. This time the fuel is the publicity being given to a report prepared at Cornell University, New York; by Professor Robert O. Pohl, who challenges the traditional wisdom that nuclear energy causes fewer health problems than result from the same amount of electricity produced from coal. The report evidently asserts that the effects of radioactive waste from uranium mines should be included in calculations of nuclear health risks. On this basis, nuclear energy is said to be between 100 and 10,000 times as dangerous as has previously been thought.

Professor Pohl is reported to be critical of calculations done by the US Environmental Protection Agency (EPA) in a 1973 report *Environmental Analysis of the Uranium Fuel Cycle*. According to the EPA's estimates, the slag heap from a mine extracting uranium to produce about 1,400 million MWh of electricity would cause 60 cases of radiation damage—mainly lung cancer—after 100 years; 95% of these cases, it was estimated, would lead to death. So an annual rate of production of 10 million MWh would result in almost 0.4 deaths after 100 years. Professor Pohl points out that the slag at the mine contains thorium-230, which—by way of radium-226—decays to (radioactive) radon-222. During the decay of the thorium (and only 0.091% will have disappeared after 100 years)

the slag will emit radon at a rate which Pohl estimates could result in a total of 396 deaths—presumably over more than 100,000 years. These calculations put nuclear energy in the same order of risk to health as coal-fired energy.

Scandinavian diary



from Wendy Barnaby, Stockholm

The report also questions the platitude that, although nuclear waste is dangerous, its volume is small enough to be controlled. On the contrary, if the slag is reckoned as part of the waste the disposal problem becomes enormous. For Sweden especially the difficulties would be immense: a ton of Swedish shale contains only 300 grams of uranium. The uranium required to produce 10 million MWh leaves 1 million tons of shale behind. Sweden's present annual electricity production is about 85 million MWh. The percentage of this provided by nuclear energy is negligible at the moment, but it is expected to rise to 12% by 1985.

Professor Pohl reportedly admits that thorium-230 and radon-222 would be formed wherever uranium occurs naturally, irrespective of whether it were mined or not. But he maintains that radon can escape more easily from the broken ground of a mine than from an undisturbed terrain. The effect of his views on the Swedish debate has yet to be seen; but it will probably provide another example of the fact that what may burst with a bang over the heads of the antinuclear lobby is, to the ears of the pronuclear government, merely a whimper.

● Experiments carried out by the Swedish Water and Air Pollution Laboratory show that the use of com-

mercial preparations labelled 'non-toxic' and sold to disperse oil leaks in water has a far worse effect on marine life than the oil itself. The results indicate that if a dispersant is used to clean up oil spills, the effect on most marine life will be more severe and last for a longer time than if no dispersant is used.

The experiments, reported in the latest edition of the Royal Swedish Academy of Sciences magazine *Ambio*, were carried out on two dispersants commonly used throughout Europe: BP 1100X (British Petroleum) and Finasol OSR2 (Fina SA). According to the Swedish researchers, normal toxicity experiments are done with adult organisms under abnormal external conditions and for a short length of time. On the grounds that such testing should be concentrated on the most vulnerable part of the organisms' life cycle, the Swedes used larvae instead. The type of larvae they worked with (Baltic herring, *Clupea harengus membras* L.) occurs naturally in the upper 10 metres of the water column, and is therefore especially susceptible to oil spills.

The tests were done in jars containing brackish sea water to which was added Venezuelan crude oil with a density of 0.868 and a total sulphur percentage of 1.9. Some of the jars also had a dispersant mixed in. A control experiment with larvae in sea water only was carried out in parallel.

In the first set of experiments, in which larvae were exposed to different concentrations of water-oil and water-oil-dispersant mixtures, the larvae reacted 50 to 100 times more strongly to a mixture of dispersant and oil than to oil alone. The larvae deteriorated by swimming abnormally, then suffering injuries and finally dying.

In the second test, larvae were exposed to mixtures of different ages (0, 24 and 72 hours). The behaviour of the larvae showed that when oil and water were newly mixed, the resulting toxicity was higher than in older mixtures of oil and water. When a dispersant was added, however, the mixture's toxicity was even higher, and it remained almost unchanged after 24 and 72 hours.

news and views

Dynamics of chemical and biological reactions

from Henryk Eisenberg

In a recent elegant study (Ben Basat and Bloomfield, *J. molec. Biol.*, **95**, 335; 1975) the kinetics of the spontaneous assembly of heads and tails of bacteriophage T4D, to form non-infective tail fibreless particles, were investigated. The authors used inelastic (or rather quasielastic) light scattering to follow an approximately 10% decrease in diffusion coefficients resulting from the attachment of the asymmetric tails to the icosahedral heads of the phage. The reaction was slow enough (the half time was about 500 s at the concentrations used) to be followed by measuring the average diffusion coefficients of the particles as a function of reaction time. The major finding was that the bimolecular rate of the reaction is about 1/500 that predicted by Smoluchovski in 1917 for a diffusion-controlled reaction between two spherical particles; the discrepancy can largely be explained by orientational factors. Careful analysis of the limits of and errors in quasielastic light scattering showed its usefulness as a tool in phage assembly studies, preferably in conjunction with other physical methods and with biological infectivity assays.

Fluctuations in time domain

Some readers may not be familiar with quasielastic light scattering. Note that one may associate with any process a 'noise' deriving from fluctuations around equilibrium states of particles in a system. Until recently it was not possible to determine the frequency spectrum of the fluctuations, nor to follow the evolution of this spectrum in time. From the time-averaged squares of the fluctuations classical elastic light scattering is derived. Fast electronic techniques and coherent monochromatic sources of radiation now make it possible to extract dynamic or kinetic parameters from the time-resolved analysis of the fluctuations.

The analysis is quite general and by no means restricted to the study of the fluctuations of scattered light. Fluctuations of fluorescence (Elson and Magde, *Biopolymers*, **13**, 1; 1974; Magde, Elson and Webb, *Biopolymers*, **13**, 29; 1974) or of electrical conduc-

tivity (Feher and Weissman, *Proc. natn. Acad. Sci. U.S.A.*, **70**, 870; 1973) in solutions, and also in nerve fibres, have been successfully examined. All that is required is that a change in the state of the system manifest itself by way of a measurable physical parameter. In technical terms the signal at a given time is compared (correlated) with itself at some previous time. In a subsequent averaging process the 'autocorrelation' function is formed. This is the Fourier transform of the spectrum and can be obtained by a simple mathematical operation. Those of us raised on the intuitive notion of the changing pitch of the whistle of an approaching or departing locomotive will easily recognise the Doppler spectrum—broadening due to the moving or reacting particle. It just so happens that modern technology often favours measurement in the time rather than the frequency domain (see *Photon Correlation and Light Beating Spectroscopy*, edit. by Cummins and Pike; Plenum, 1973; Chu, *Laser Light Scattering*; Academic, 1974; Berne and Pecora, *A. Rev. Phys. Chem.*, **25**, 233; 1974). A very broad range of phenomena has conveniently become accessible, in an experimental range having time constants corresponding to frequencies between 1 and 10^8 Hz.

In the study of the kinetics of macromolecular association and transconformation reaction two cases may be distinguished. The first case, which is also the topic of the Ben Basat and Bloomfield paper, focuses on the situation in which the reaction rate is slow compared to diffusion. If reactants and products have different translational diffusion coefficients, the scattered spectrum or autocorrelation function is that of a polydisperse system and can be resolved to provide concentrations of the various participants in the reaction. If the time of measurement is fast compared to the time of the reaction, the evolution of the latter is a function of time, and therefore the kinetic constants can be evaluated. This is what Ben Basat and Bloomfield have successfully done in the T4D phase head-tail assembly system.

The second case considers a reaction in dynamic equilibrium, in which the

reaction rate is greater than, or comparable to, the diffusion rates for the reacting species. In this case theory predicts that the time autocorrelation function measured will be a sum of exponentials, whose decay constants are functions of both diffusion and reaction rates. However attractive this prediction may sound, conflicting and largely unsubstantiated claims have in the past resulted from studies of this kind. Decay curves of the autocorrelation function of reacting species are usually too complex to be resolved in a simple sum of exponentials in a unique, meaningful, way. But more recently Uzgis and Golibersuch (*Phys. Rev. Lett.*, **32**, 37; 1974) reported a decay term independent of scattering angle in scattered-light intensity fluctuations from haemoglobin solutions. They attribute these fluctuations to the haemoglobin association-dissociation reaction; variations in experimental conditions affect the excess fluctuations in a predictable way.

Variations in method

Other examples exist, not necessarily related to the analysis of scattered light. In their very imaginative study, Feher and Weissman applied a constant voltage across an aqueous solution of an electrolyte, beryllium sulphate, in ionisation and hydration equilibrium. Fluctuations, on a fast time scale, occurred around the equilibria positions, and the reactants carried unequal charge. Therefore strong time-dependent fluctuations were observed in the ionic conductance and it was possible to measure the autocorrelation function of the conductance and derive meaningful kinetic constants for the ionisation process. These were in good agreement with results earlier obtained by the more perturbative temperature jump method. In another pioneering study Elson and his collaborators took advantage of the fact that the fluorescence of a dye, ethidium bromide, is strongly quenched upon binding to DNA. Once more fluctuations of binding (hence of fluorescence) occurred around the equilibrium state of the reaction. If a suitable exciting laser light was now focused on a tiny volume of the reac-

tion mixture, the concentration of fluorescing particles could be studied as a function of time and the autocorrelation function formed. The kinetic constants derived again were in good agreement with relaxation spectroscopy results.

In variety there is spice. New information can be derived from experiments in which external fields are imposed on a fluctuating system at thermal equilibrium. Ware and Flygare used electrophoretic scattering (*J. Coll. Interf. Sci.*, **39**, 670; 1972) in which charge-carrying scattering particles (in random Brownian motion) acquire and additional linear momentum by an applied d.c. electric field. In this situation the spectrum, or the autocorrelation function, can be clearly decomposed into contributions coming from the random, Brownian motion, and from the electrophoretic mobility of the particles. The resolution of the method is thus that, in principle, the motion of different particles, moving with different velocities, can be clearly distinguished. Hydrodynamic and sedimentation fields can, in principle, also be applied. In recent applications, Mustach and Ware (*Phys. Rev. Lett.*, **33**, 617; 1974) reported on the study of protoplasmic streaming by laser light scattering and Josefowicz and Hallet (*Appl. Optics*, **14**, 740; 1975) described a sophisticated electrophoretic light scattering assembly suitable for analysis at low sample concentrations.

Clegg, Elson and Maxfield (*Biopolymers*, **14**, 883; 1975) introduced a new method in which a reaction is perturbed by repetitive application of a small pressure change. Reaction progress is followed by monitoring optical properties such as absorbance, fluorescence or light scattering. Phase-sensitive detection methods are used to enhance the low signal-to-noise ratio resulting from the slight perturbation. Yet the slight perturbation allows the analysis of closely spaced microstates, whereas the conventional perturbation methods, such as temperature-jump, often cause the system to transverse many intermediate conformations in the course of a single observation. This is of particular importance in reactions that involve the folding of biological macromolecules, in processes involving a multitude of partially ordered intermediate conformations.

In conclusion, a number of new tools for the study of reaction rates in chemical and biological reactions are being developed. In these new techniques advantage is taken of the spontaneous fluctuations around equilibrium states in the systems under study and, in other instances, only minor perturbations are superimposed on these natural fluctuations. □

Piezoelectric polyvinylidene fluoride

from Paul Calvert

THERE has recently been considerable interest in the development of piezoelectric (producing a voltage when stretched) polymer films. The current champion is polyvinylidene fluoride (PVDF $(\text{CH}_2\text{-CF}_2)_n$) which has a piezoelectric modulus d_{31} of $1\text{--}2 \times 10^{-7}$ c.g.s.-e.s.u. compared to about 18×10^{-7} for barium titanate. The polymer, having the advantage of being available as a flexible film, is ready for use in microphones and possibly in loudspeakers. The origin of the piezoelectric effect in PVDF is still unknown and the matter is considerably confused by the complicated phase behaviour of this polymer. A pair of papers by Murayama and coworkers (*J. Polymer Sci. Physics Edition*, **13**, 929 and 946; 1975) of the Kureha Chemical Company in Japan, one of the two commercial producers of this polymer, go a long way towards clarifying the question if not to producing answers.

In 1969 Fukada (*Jap. J. appl. Phys.*, **8**, 960) and Kawai (*ibid.*, 975) showed that a piezoelectric effect could be produced in stretched and oriented films of several polymers when they were poled by applying an electric field of 500 kV cm^{-1} across the thickness of the film. When poled most polymers acquire a stable surface charge and are known as electrets. Except for PVDF there is no piezoelectric effect if the polymers are unoriented (Cohen and Edelman, *J. appl. Phys.*, **42**, 3072; 1971). This seems reasonable because the orientation process will tend to produce extended chain conformations of the polymer and in the vinyl polymers this would result in alignment of the permanent dipoles such as that of the $-\text{CF}_2-$ group in PVDF. If the electric field caused these to point preferentially in one direction the material could be piezoelectric.

What makes PVDF special is its phase behaviour. There are three crystal forms: I, also known as β , II or α , and III, each of which can be produced by precipitation from a suitable solvent. Cooling from the melt gives form II which converts to form I on stretching. In form I the chains are extended so that the dipoles within one chain are parallel and the chains are packed into an orthorhombic lattice so that the dipoles on individual chains are parallel (Lando *et al.*, *J. Polymer Sci.*, A-1, **4**, 941; 1966). Obviously this form should be piezoelectric if it is properly oriented. Doll and Lando (*J. macromolec. Sci. Phys.*, **2**, 205; 1968) showed that copolymerisation of tetra-

fluorethylene ($\text{CF}_2=\text{CF}_2$) with the vinylidene fluoride promoted formation of phase I and suggested that the 'head to head' groups $(-\text{CH}_2\text{CF}_2\text{CF}_2\text{CH}_2)$ normally present at 5–10% in PVDF promote form I. Tadokoro *et al.* (*Macromolecules*, **8**, 158; 1975) go into this in more detail with infrared and Raman spectra and discuss the structures of form II, where the dipoles cancel, and form III which is similar to form I.

Hayakawa and Wada (*Adv. Polymer Sci.*, **11**, 1; 1973) have discussed the possible origins of piezoelectric effects in polymers and distinguish between intrinsic effects due to oriented dipoles and extrinsic effects due to trapped charges. On the face of it the former looks more likely for PVDF where the piezoelectric modulus increases with the content of form I whether produced by stretching or copolymerisation. Since the dipoles in form I are aligned it is simply necessary to assume that the crystals realign in the electric field or that more form I arises during the poling.

One observation that clashes with this is that of a negative piezoelectric modulus d_{33} in the film thickness direction by Burkard and Pfister (*J. appl. Phys.*, **45**, 3360; 1974). Any simple dipole model would predict a positive value; that is the film should get thicker when a field is applied parallel to the original poling field.

Murayama *et al.* report measurements on the decay of surface charge after poling. PVDF electrets have a charge of the same polarity as the field (homocharge) which is probably due to charges injected from the electrodes. This decays over a few hours to leave a charge of opposite sign (anomalous heterocharge) which is stable, even above the poling temperature. This anomalous heterocharge is correlated with, but not the origin of, the piezoelectric effect. They also observe that if a polyester film is placed between the positive electrode and the PVDF during poling the piezoelectric modulus and heterocharge are much reduced; between the negative electrode and the film it has little effect. No evidence is found for the production of more form I on poling and they argue that re-orientation of existing crystals is very unlikely. Thus they conclude from their observations that the piezoelectric effect cannot be due to permanent dipoles but must be caused by the local migration and trapping of charges in the form I crystals. This process is somehow associated with charge injection from the positive electrode. The matter cannot really be considered settled until more is known about the nature of the induced dipoles and why form I in PVDF is particularly suitable. By then there will probably be a new champion polymer. □

Deuteron-gamma angular correlations

from P. E. Hodgson

WHEN deuterons are inelastically scattered by nuclei they leave the target nucleus in an excited state, which usually decays by gamma emission. The angular correlation between the inelastically scattered deuteron and the subsequent de-excitation gamma ray can provide important information about the mechanism of the reaction.

An illustration of this has recently been provided by the work of Scheib, Hofmann and Vogler (*Phys. Rev. Lett.*, **34**, 1586; 1975) on the inelastic scattering of 10 MeV deuterons by ^{24}Mg . The angular correlation was measured for gamma rays emitted in the reaction plane, and in this case the correlation function is given by

$$W(\varphi_\gamma) = A + B \sin^2(\varphi_\gamma - \varphi_1) + C \sin^2(\varphi_\gamma - \varphi_2)$$

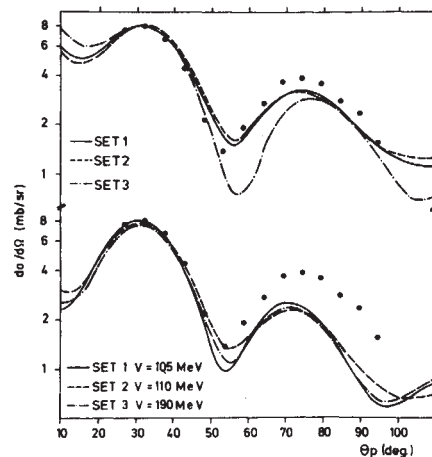
where φ_γ is the angle between the beam direction and the angle of emission of the gamma ray, and A , B , C , φ_1 and φ_2 are constants whose values are obtained by fitting the experimental data. The constants A , B , and C , are related to products of the reaction substate amplitudes, which may be calculated from a detailed theory of the reaction. This makes the correlation function much more sensitive to the details of the reaction than is the differential cross section, which is given by the sum of the absolute squares of all the reaction amplitudes. The sensitivity allows the correlation function to tell us more about the details of the reaction mechanism than we could learn from the differential cross section alone.

This is strikingly shown by the ^{24}Mg results. The figure shows the differential cross section compared with calculations using two different models of the reaction. The upper curves show the results of distorted wave calculations with three different sets of distorting potentials and assuming that the reaction takes place in a single step from the ground state to the 2^+ excited state. The lower curves show similar calculations that also take into account the coupling to the 4^+ state, so that some of the excited 2^+ states are formed by first exciting the 4^+ state directly, followed by de-excitation to the 2^+ state. All the calculations used the symmetric rotator model including spin-orbit interactions.

When comparing such theories with the experimental data we look for a good fit to the main forward peak, which is given by both theories and all potentials, together with a qualitative agreement with the rest of the cross section. The angle and width of the second peak are given quite well but its magnitude is somewhat underestimated by all calculations, rather more so by the theory including the two-step

process than by the one-step theory. All these fits are acceptable, but do not distinguish between the two reaction mechanisms.

When we examine the deuteron-gamma correlation data the comparison is quite



Differential cross section for the inelastic scattering of 10 MeV deuterons by ^{24}Mg with excitation of the lowest 2^+ state compared with distorted wave calculations with three sets of distorting potentials using the one-step theory (upper curves) and the two-step theory (lower curves). V is the depth of the real part of the distorting potential.

different. If the results for the parameter C in the above expression for $W(\varphi_\gamma)$ are compared with the results of the six calculations already mentioned, the greater sensitivity of the correlation function is obvious. The three calculated curves for the one-step theory differ markedly and none fits the data, even qualitatively; however two of the calculations for the two-step theory are in good qualitative agreement with the data, and in particular give the deep minimum found around 55° . These two potentials are quite similar, and correspond to the deuteron potentials known to be physically realistic by calculations based on the nucleon-nucleus potential and using the folding model. The third potential for the two-step theory is physically unrealistic and also fails to give a good fit to the angular correlation data.

It is of course well known from many other studies that two-step processes must be taken into account in a detailed treatment of inelastic scattering, and this is confirmed by the correlation analysis. What is new is the striking way that it definitely excludes the one-step process as a sufficient explanation; this shows that the correlation analysis could be a powerful way of determining the reaction mechanism in cases where it is not already known. □

Tunable alkali halide lasers

from John Walker

TUNABLE lasers, particularly dye lasers, now cover the whole of the visible spectrum, and are currently revolutionising optical spectroscopy. Unfortunately dye lasers do not operate in the near infrared, a region of great importance in molecular spectroscopy, pollution detection and fibre optic communications. Hence the significance of a recent paper by Mollenauer and Olson (*J. appl. Phys.*, **46**, 3109; 1975) who have demonstrated tunable laser action in potassium chloride, and have suggested that the wavelength range 0.9 to $3\ \mu\text{m}$ could be covered by suitable choice of alkali halide. These compounds, so important in our understanding of solid state physics and of point defects, have at last found a practical application.

Laser action over the range 2.5 to $2.9\ \mu\text{m}$ was achieved using the $F_A(\text{II})$ centre in lithium-doped potassium chloride pumped by a krypton ion laser at 647.1 nm. An input power of 40 mW was necessary when the KCl crystal was at 77 K, and lasing could be induced at temperatures up to 200 K.

The operation of the laser can be explained as follows. The $F_A(\text{II})$ centre consists of a halide vacancy (an F centre) trapped by an impurity

metal ion—in this case lithium occupying a potassium lattice site. The centre is excited by the pump laser and then relaxes by rearrangement of the chloride ions around the vacancy. A population inversion is thereby achieved between the relaxed excited state and the unrelaxed ground state, which is emptied by further atomic rearrangement into the relaxed ground state. The system is therefore an ideal four-level laser.

Similar arguments apply to the F and the $F_A(\text{I})$ centres, but in their case the excited state relaxation is very small, and there are competing de-excitation processes, making them unsuitable for laser action. However, two other potentially useful defects are the F_2^+ centre (a pair of adjacent halide vacancies), and the $F_B(\text{II})$ centre (similar to the $F_A(\text{II})$, but with two impurity atoms). These centres should enable a total tuning range of 0.9 to $3\ \mu\text{m}$ to be achieved, and Mollenauer and Olson are currently trying to construct an F_2^+ centre laser using KCl.

The combination of simplicity, tunability, low pump power and potential frequency stability makes alkali halide lasers a promising prospect in the near infrared.

HL-A, the major histocompatibility system of man, which has its analogue, *H-2*, in the mouse, and also in many other species, is now known to contain many genes controlling cell surface determinants, immune response differences, some components of the complement system (in man C3 proactivator (Bf), C2 and probably C4) and perhaps other related functions connected in general with cell-cell recognition. The *HL-A* system was first defined by a series of antigenic specificities—identified by serological techniques using peripheral blood lymphocytes—which were assigned to two separate series (LA and FOUR) corresponding to two closely linked loci, now called *A* and *B* respectively, with multiple alleles. Although matching for transplantation using these antigens, which was the original impetus behind their study, has turned out to be more difficult and complex than was hoped, knowledge of the wide range of important functions controlled by genes in the region has grown from these original serological studies. The earliest data on the recombination fraction between the *A* and *B* loci already indicated that the *HL-A* region probably included very many genes, at least hundreds, if not a few thousand.

The development of the *HL-A* system has been greatly stimulated by a series of international collaborative workshops started by D. B. Amos of Duke University, North Carolina, in 1964. These workshops, which involved exchange of reagents among a large number of participating laboratories and the combined analysis of the resulting data, have each been major turning points in the development of knowledge of all aspects of the *HL-A* system. Thus, the second and third workshops organised by J. J. van Rood of the University of Leiden in 1965 and by R. Ceppellini of the University of Turin in 1967, placed the definition of the first described antigens on a firm footing and established that they all belonged to a single system of closely linked genes. The fourth and fifth, organised respectively by P. Terasaki of the University of California, Los Angeles in 1970, and by J. Dausset of the University of Paris in 1972, established that two linked loci control the main serological specificities and, through a world-wide series of population studies, the universality of this genetic model and the general distribution of the antigens among the various major human population groups.

The aims of the sixth workshop organised by F. Kissmeyer-Nielsen of the University of Aarhus, culminating in the meeting in Aarhus, were the definition of specificities identified by the mixed lymphocyte culture (MLC) test and the improved serological de-

Histocompatibility testing international

from W. F. Bodmer

The 6th International Workshop on Histocompatibility Testing was held in Aarhus, Denmark, on June 29–July 5.

finition of specificities of the more recently described *C* locus, as well as of newer specificities of the *A* and *B* loci. The MLC reaction is measured by the proliferative response when cultured together of lymphocytes from different individuals.

A WHO nomenclature committee, which met immediately after the workshop, recommended that with the increasing number of loci being defined in the region, *HL-A* should be the name given to the whole region, while the individual loci within it will be called *A* (formerly the LA or first locus), *B* (formerly the FOUR or second locus), *C* (formerly AJ or the third locus) and *D* for the locus controlling the determinants identified by MLC typing. The probable order of these four loci is *D, B, C, A*. New loci of the *HL-A* region will be similarly named as they become clearly identified.

MLC typing

Following the discovery by F. Bach (of the University of Wisconsin) and D. B. Amos in 1967 that at least the majority of MLC reactions were controlled by genetic differences in the *HL-A* region, further work by Amos and others established the fact that the determinants of this reaction were controlled by one or more loci that were genetically separable from *A* and *B*, although closely linked to them. Subsequently, a number of laboratories clearly established that it was possible to use MLC reactions to a set of specially chosen lymphocytes to define MLC determinants, namely to do 'MLC typing'. For this the one-way test is used (in which one of the two sources of lymphocytes to be mixed is inactivated, for example by X rays or mitomycin, so it can only act as a stimulus for the other, which is not inactivated) and stimulators are chosen from individuals known to be homozygous for the *HL-A* region, usually on the basis of family studies. Because of the high level of polymorphism for the *HL-A* system, such homozygous typing cells are most readily found amongst the offspring of parents who are related. If a test cell does not show an MLC response to a given homozygous typing cell, then it presumably has the determinant carried by the typing cell. Such reactions to a series of typing

cells can be analysed following the same principles used for the analysis of serological reactions, to establish specificities defined by similar reactions to a set of typing cells. For the workshop, fifteen collaborating laboratories from the United States and Europe, guided by E. Thorsby of the University of Oslo, each used the same 62 carefully chosen representative homozygous typing cells to type a panel of up to 100 responders.

The resulting data, which undoubtedly represent the most extensive so far collected on MLC determinants, were coded in a standard format by each of the laboratories and analysed centrally by A. Piazza of the University of Turin, with the help of L. Lamm and F. Jorgensen of the University of Aarhus. There are many statistical problems involved in the assessment of MLC reactions and in the definition of positive and negative responses which were clearly revealed by this combined analysis. The availability of such a large body of data, however, was a great help in the more or less clear cut definition of a series of eight determinants of the *HL-A-D* locus, which were seen to correspond to various determinants previously defined by one or more different laboratories. Each *D* locus specificity could be identified by a cluster of up to six different typing cells which, for the most part, reacted together when the corresponding determinant was present on a responder cell. Family and population data suggested that these six determinants behaved as if controlled by a series of alleles at the *D* locus, although it is clear that, as in the case of the serologically defined specificities of the *A*, *B* and *C* loci, new data may lead to 'splitting' of these MLC determinants into two or more sub-specificities, and indeed to splitting of the *D* locus itself. A particular feature of the *D* locus determinants, which was clearly confirmed by the workshop, was their tendency to be in strong linkage disequilibrium with *B* locus alleles. Linkage disequilibrium is the tendency for alleles at different loci, usually closely linked, not to be associated at random in a population. It is in general the main cause of population associations between functions controlled by linked genes, and so is the probable basis for most of the known *HL-A* and disease associations which have been interpreted as due to linkage disequilibrium between alleles of the *B* locus and alleles at a linked immune response locus. Thus, it is, for example, the DW2 specificity (formerly LD 7a), which is closely associated with the serologically defined specificity B7 (formerly HL-A7) and also very significantly increased in frequency among patients with multiple sclerosis.

Serology

The serological part of the workshop was based, as before, on an exchange of carefully selected sera among the participating laboratories. Each of the 73 laboratories, including one or more from every continent (except Antarctica) typed up to 250 cells with the same set of 178 sera. The populations of cells included the laboratories' own well-typed panels, some families, some 'exotic' population groups and also, in the United Kingdom as a convenient source of B lymphocytes, lymphoblastoid cell lines and cells from patients with chronic lymphocytic leukaemia (CLL).

The combined analysis of this large body of data, which was organised by Julia Bodmer of Oxford University with the help of Piazza and others, first of all clearly establishes the definition of five specificities of the *C* locus. Specificities of this locus, whose existence was first suggested by L. Sandberg of the University of Goeteborg and E. Thorsby and their collaborators in 1970 and which was later confirmed by A. Svejgaard, University of Copenhagen, and others, have so far been more difficult to define than those of the better known *A* and *B* loci. This is, in large part, because alleles of the *C* locus tend to be in strong linkage disequilibrium with those of the *B* locus and so easily confused with them. Eighteen antigens of the *A* locus and twenty-four of the *B* locus were recognised by the workshop sera. These included better definition of a number of previously suggested specificities, often 'splits' of already defined antigens. This splitting of antigens is a common phenomenon in HL-A serology mainly as a result of the existence of cross-reacting families of antigens which are seldom at first separated by the available antisera.

Ia-type specificities

A most important byproduct of the workshop was the simultaneous description by at least nine different laboratories of various approaches to the serological identification of Ia-type specificities. In the mouse the genetic region between the *H-2K* and *H-2D* loci (which correspond to *HL-A-B* and *HL-A-A* respectively) has been shown to contain, in addition to genes controlling immune response, further genes controlling a new set of serological specificities called *Ia* for immune associated. Unlike the *K* and *D*, or *HL-A-A*, *B* and *C* antigens, the *Ia* antigens have a tissue distribution that is relatively specific, including especially *B*, but excluding *T* lymphocytes. Similar antigens were first described in man by van Rood and coworkers using inhibition of the MLC reaction as a screen and then immunofluorescence of

peripheral blood B lymphocytes. Other approaches to the identification of human Ia-type sera include in particular the use of B-derived lymphoblastoid cell lines and CLL cells, as well as partially purified peripheral blood B lymphocytes and immunisation between unrelated individuals identical at the *HL-A-A*, *B* and *C* loci. Ia-type antibodies are quite commonly found in the usual *HL-A-A*, *B* and *C* locus typing sera (which are mostly obtained from multiparous women who make these antibodies as a result of foetal-maternal stimulation) and were shown by W. F. Bodmer of the University of Oxford and H. Dick of the Glasgow

Royal Infirmary and their collaborators to be present in just under one-third of the workshop sera. Several reports indicated, as might be expected, close association between Ia-type serological reactions and MLC determinants of the *D* locus. Clearly, serological Ia typing is much easier than MLC typing and so may become a powerful tool in disease association and other related studies. There is also the definite possibility that Ia specificities will be of greater direct importance for the problem of clinical transplantation than are the specificities of the *HL-A-A*, *B* and *C* loci.

This workshop was as exciting and

Hydrous minerals in meteorites

from David W. Hughes

THE water content of rocks, whether they be terrestrial or meteoritic, can provide an important clue to their place of origin. Meteoritic minerals are nearly always fresh and undecomposed—which contrasts with many of the water-containing products of weathering and erosion which occur in such profusion on Earth. By studying the chemical composition and paragenesis (the groupings of different types of minerals) of meteorites it is found that in the main they must have crystallised from a fiery melt which had an exceptionally low water content in contrast to the wet volcanic terrestrial melts which solidify to form igneous rocks. Only in type 1 and 2 carbonaceous chondrites is water reasonably abundant. These were formed at low temperatures and have not been subsequently heated above 350 °C. They are thought to have compositions very close to that of the primordial dust which coalesced to form the Solar System and they contain many primary volatile components. These meteorites are thought to be parental to other types which are formed by complicated thermal evolution processes and geochemical depletion reactions.

Ashworth and Hutchison in this issue of *Nature* (page 714) report having found hydrous minerals in the meteorite Nakhla, a diopside-olivine achondrite which fell in Egypt in 1911 and in the meteorite Weston (a chondrite which fell near Weston, Connecticut in 1807). Both are meteoritic types in which water is rare.

The water in Nakhla and Weston is in the form of iddingsite, a mixture of hydrothermal alteration products of olivine ((Mg, Fe)₂SiO₄). The iddingsite is found as red-brown veins along cracks in the olivine. These veins were probably formed by shock deformation, and water present at this time perco-

lated down the cracks and caused mineral alteration.

Ashworth and Hutchison conclude that magmatic water must have been present during the formation of this shocked olivine, and that the meteoritic mineral originated on a body in the Solar System that had a hydrous atmosphere. There is however still the possibility that the meteorites were 'dry' when they left the parent body and that they picked up the water later. The water could also have come from an admixture of some carbonaceous chondritic material in with the meteorite; this however has not been chemically identified. The interaction between solar wind ions and meteoritic minerals causing hydrogen implantation is discounted because lunar breccias, which have been exposed to the solar wind for aeons, are generally anhydrous. That leaves terrestrial weathering as the alternative source of water. Was the hydrous mineral produced by the hydrolysis of primary lawrencite (FeCl₂) by water vapour in the terrestrial atmosphere? As Weston contains an order of magnitude more water than Apollo 16 rocks this requires a very high initial content of chlorine. Both Nakhla and Weston were observed falls and were collected soon after hitting the Earth. However as Nininger (*Out of the Sky*, Dover, 1952) points out, we never see a truly unweathered meteorite, the atmosphere through which they fall being nearly saturated with water—to say nothing of the air in the vicinity of the museum shelf on which they have been sitting for over half a century.

So the mystery remains—did the meteorites come from a wet planet or have they picked up their water later? It would be heartening to think that Earth has not been the only wet place in the life of the Solar System.

stimulating as its predecessors, reflecting the enormous effort devoted by Kissmeyer-Nielsen and his associates to its organisation and showing once again the value of such world-wide collaborative studies. The next workshop will be organised in Oxford in about 2 years' time and will have as its main theme the definition of human Ia specificities, their relation to MLC determinants and their significance for disease association and clinical transplantation. □

Reliability of molecular phylogenetic trees

from David R. Thatcher

In principle a comparison of the primary structure of the same protein from a number of related organisms should provide evolutionary information of high precision. The amino acid sequence of a protein is a translated copy of the sequence of nucleotide bases in the structural gene and therefore is the one phenotypic character that is a direct reflection of the structure of the genome. The comparative morphology of amino acid sequences should therefore permit the deduction of some of the discrete mutational steps which have occurred during the evolution of an organism. Providing no genetic transfer has occurred and the gene has retained the same function throughout its history, a phylogenetic topology derived from amino acid sequence comparisons should represent the true ancestral relationships of the organisms expressing that particular gene. Molecular phylogenetic methods have the potential advantage of being able to distinguish evolutionary relationships where the classical methods of evolutionary biology do not produce a convincing answer, for example where the fossil evidence is controversial or absent or where there is little morphological diversity to compare.

In practice little new phylogenetic information has been derived from the construction of trees based on molecular evolutionary data and the method has received a somewhat hostile reception from other evolutionary biologists (these points are fully discussed in the excellent review of J. Williams in *The Chemistry of Macromolecules: Proteins*; Medical and Technical Publishers, 1974). The most damaging criticism proposed is that data based on a single structural gene cannot possibly reflect the evolution of the whole genome. Nevertheless, trees constructed by comparing sequences of vertebrate cyto-

chromes *c*, haemoglobins and fibrinopeptides are each more or less in agreement with the accepted phylogeny for this group, showing that the method at least works even if its precision is not as great as at first hoped.

The resolution of the sequence method is unquestionably restricted and if future projects are going to be attempted with an eye to solving particular phylogenetic problems, these limitations must be fully defined. These limitations are due to two main factors. First, to what extent is the assumption of parsimony justified at the molecular level? There is no absolute way of knowing the degree to which parallel, back or convergent mutations affect observed amino acid similarities. Second, if one accepts the most parsimonious solution as being the actual evolutionary history of a gene, the numerical problems involved in calculating this solution for even a small number of alternative topologies is formidable.

Peacocke and Boulter (*J. molec. Biol.*, **95**, 513; 1975) have begun to assess the extent to which these technical limitations have affected their conclusions on higher plant evolution. The evolution of a protein was simulated in a computer and the reconstruction of this hypothetical phylogeny was attempted using two different methods. The accuracy of each procedure was then estimated by determining the number of times a branch had to be moved in order to restore the original topology. The two methods employed were the ancestral sequence method of Dayhof (*Atlas of Protein Sequence and Structure*, 1972) and the matrix method of Moore, Goodman and Barnabas (*J. theor. Biol.*, **38**, 423–457; 1973). The ancestral sequence method infers the structure of nodal ancestral sequences as it generates a tree by adding one sequence at a time to the topology. New branches are added with respect to the most parsimonious sequences (real and ancestral) already in the tree. In the matrix method a distance matrix is calculated and an approximate tree is produced by successive pairwise clustering. This tree is then adjusted until hypothetical mutational distances (computed from the tree by comparing the mutational distances of two neighbours with the average mutational distance of all other members of the tree) approach the actual values of the original distance matrix. Applied to the model data the accuracy of the ancestral sequence method became progressively worse as the number of observed substitutions in the data set was increased. In contrast the matrix method, although less accurate than the ancestral sequence method at lower degrees of substitution, improved as the

variation between the sequences increased. Phylogenetic trees were then constructed from model data which varied to the same extent as the real data Boulter's group has accumulated on higher plant cytochrome *c*. The error observed in tree building with the model data and the ancestral sequence method ranged between 2% and 8% whereas the matrix method operated with an error of 9% to 15%. For a rapidly evolving protein like plastocyanin methods were equally successful in predicting the correct topology (error 8% to 12%).

Before molecular phylogenies are considered seriously by systematicists, some attempt must be made to quantify the errors inherent in each method; from the extent to which tree topologies may be influenced by sequencing mistakes to the probability of a given tree being the most parsimonious solution possible. Peacocke and Boulter have shown that an optimal amount of amino acid variation is required for the most accurate topological assignments by the ancestral sequence method. As structural genes have characteristic rates of evolution, which vary greatly from gene to gene, only a limited range of accurate phylogenetic information can be obtained from the study of a single gene.

Convincing evidence is obtainable but only by investigating the best gene for a particular phylogenetic problem and applying the numerical method most suited to the data. □

Consequences of hydrothermal circulation

from Peter J. Smith

LAST year, Lister (*Eos*, **55**, 740; 1974) made a strong plea for the testing of the hydrothermal circulation hypothesis using deep sea boreholes near oceanic ridges. His argument was that there is growing evidence that certain observations, notably in the fields of oceanic heat flow and ocean floor mineralisation, can only be explained in terms of the penetration of water to substantial depths (possibly several kilometres) in the new crust forming at ridge crests.

Presumably it is still too soon to expect any results to have emerged from such a proposal. In the meantime, however, indirect support for hydrothermal circulation continues to accumulate. Lee and Von Herzen (*Geophys. Res. Lett.*, **2**, 201; 1975), for example, have now reported 15 new heat flow measurements from the South Atlantic triple junction near 55°S, 0°E—the zone in which the boundaries of the African, Antarctic and South

American plates meet. Although the data are few, they show a distinct geographical correlation; on ridge crests flanking axial valleys the heat flow is high, in the depressions of fracture zones and axial valleys it is intermediate to low and on ridge flanks it is low. At one extreme, four values higher than $4.0 \mu\text{calorie cm}^{-2} \text{s}^{-1}$ were observed on the crests of three ridge segments, whereas at the other, a value of $0.4 \mu\text{calorie cm}^{-2} \text{s}^{-1}$ was found in a depression of an axial valley near its intersection with an adjacent fracture zone.

The problem with these and previous similar results from less complex areas is that they conflict with the conditions of steady state heat conduction, by which heat flow should be higher through depressions and valleys than through ridges. Variations in the thermal conductivity of the sediments in which the heat flow was measured would be one possible way of reconciling observation with conduction theory, but in the case of the South Atlantic triple junction such variations do not exceed 50% whereas heat flow values vary by at least an order of magnitude.

Lee and Von Herzen thus favour an explanation involving hydrothermal circulation in the cracks produced by thermal and/or tectonic tensional stresses near the spreading axis. According to this view, which is apparently the only one offering any hope at present, the high heat flow over a ridge crest reflects the topographically controlled ascending limb of the circulation system in the permeable basement rocks (although it could result from conductive cooling of the crust following the sealing of cracks generated at the spreading centre). The intermediate to low values in the axial valleys may then be attributed to the circulation's descending limbs. Similar values in fracture zone depressions are not quite so easily explained in detail, although reasons for cracking and subsequent hydrothermal circulation there are not difficult to envisage.

The only real difficulty is in accounting for the low heat flow over ridge flanks. One possibility is that the irregular topography here supports a locally controlled hydrothermal system which imitates the wider pattern. Thus hot water would ascend through elevated terrain and descend in depressed regions. The observed low heat flow would then result from a known sampling bias towards local sediment-filled topographic lows. A second possible explanation is that secondary circulation occurs in cracks reopened during the dehydration of rocks previously hydrated near the spreading axes. But either mechanism affirms the crucial role of hydrothermal circulation.

A similar conclusion has also been reached by Piper *et al.* (*Earth planet. Sci. Lett.*, **26**, 114; 1975) but in a quite different context. Piper and his colleagues have analysed (for major, trace and rare earth elements and uranium isotopes) an iron-rich (about 30%) deposit dredged from the upper flank of Dellwood Seamount in the north-east Pacific. What attracted them to the sample in the first place was its iron-richness, because oceanic deposits with high iron contents (20–35%) and high Fe/Mn ratios (3–75) have previously been associated with active ridge spreading. Indeed, in overall composition and mineralogy the new sample closely resembles other iron-rich deposits attributed to volcanic hydrothermal activity. But do the separate elemental compositions and distributions also support such an origin?

As Dellwood Seamount is close to the North American continent, an obvious possibility is that some of the elements in the sample are terrigenous, introduced into the oceans by rivers. This view may be rejected, however, partly because the major element composition differs from that of metal deposits from hemipelagic environments and partly because the Th content and Th/U ratio are much lower than those of typical river muds. Are, then, any of the elements derived from sea water? The fact that the rare earth element pattern and the $^{234}\text{U}/^{238}\text{U}$ ratio differ significantly from those of normal sea water suggests that this is unlikely, at least for the rare earths and uranium

isotopes (and probably for others too). Indeed, although the absolute concentrations of rare earth elements are much lower than those in oceanic sediments and basalts, the pattern of these elements is almost identical to that of ridge basalt.

Taken together, these observations can only be explained on the assumption that the unusual composition of the iron-rich deposit derives from a suboceanic source. And the only known way in which this source may be tapped is by hydrothermal circulation which leaches out the metals from flow interiors and transports them to the surface. Admittedly there is a problem. The $^{234}\text{U}/^{238}\text{U}$ ratio is not only much higher than that of normal sea water, it is also much higher than that generally found in iron deposits now forming near known active volcanic centres. In the case of the Dellwood deposit it is therefore also necessary to assume that ^{234}U is leached from the rock preferentially. The point is, however, that even this anomaly can be accommodated within a hydrothermal system. □

Constructing semisynthetic polypeptides

from D. G. Smyth

A SEMISYNTHESIS is one that utilises a fragment of a natural substance as a prefabricated unit in the construction of a larger molecule. The term does not include the preparation of simple derivatives of a natural protein nor of course does it cover the chemical synthesis of peptides from their building bricks, the constituent amino acids. The hallmark of a semisynthesis is that it involves the combination of two components, the one a fragment of a natural substance, the other a product formed by chemical synthesis. This description would include the preparation of ribonuclease S by association of the subtilisin-produced S-protein (residues 21–124 of ribonuclease) with a synthetic S-peptide (corresponding to residues 1–20). The combination of natural insulin A-chain with synthetic B-chain, or *vice versa*, would also qualify as a semisynthesis, as would the conversion of porcine insulin to human insulin by replacement of the B-chain octapeptide.

In the field of peptide synthesis, the idea that an intermediate fragment might be obtained by excision from a protein of known structure is attractive. Such fragments can be obtained in pure form and without racemisation by the action of proteolytic enzymes;



A hundred years ago

IN the Paris International Maritime Exhibition there is a small object deserving of notice. It is a platinum wire placed in a bottle and ignited by electricity from a bichromate battery. It is intended to be immersed in the sea, and the light emanating from it is said to attract an immense number of fishes. Experiments have been tried lately on the coast of the Côtes du Nord department with a fishing-boat, and have proved very satisfactory, on a bank of sardines. The glass must be green or black, otherwise the fish are frightened by the glare and do not follow the submarine light.

from *Nature*, **12**, 388; Sept. 2, 1875

but difficulties arise in activating the fragment for further use. This is illustrated in a semisynthesis of human β -MSH by Burton and Lande (*J. Am. chem. Soc.*, **92**, 3746; 1970), utilising the porcine hormone as an intermediate. Human β -MSH possesses four more amino acids than porcine β -MSH and a tetrapeptide was therefore synthesised with the intention of extending the NH_2 -terminus of the porcine molecule. Unfortunately porcine β -MSH has two NH_2 groups, the α - NH_2 group at position 1 and the ϵ - NH_2 group at position 6, and the coupling reaction led to the formation of products with the tetrapeptide attached at more than one site. Inevitably the yield of the required α -linked hormone was low. In semisynthesis it is clearly important that, before coupling, all reactive side chains should be protected to ensure that only terminal residues are free to participate in the formation of peptide bonds. Herein lies the difficulty.

Offord (*Nature*, **221**, 37; 1969) has reported that carboxyl groups in peptides can be protected under mild conditions by reaction with phenyldiazomethane. This forms benzyl esters at the side chains of all aspartic and glutamic acid residues but it also esterifies the C-terminal carboxyl group; the latter, however, can be regenerated selectively by the action of an esterase. When the fragment is initially obtained from a natural polypeptide by the action of trypsin, the same enzyme can be used to remove the C-terminal benzyl group. Similarly NH_2 groups can be reversibly blocked in a precursor polypeptide before the required fragment is released by proteolytic cleavage. Provided the peptide was initially present within the sequence of the precursor and not at the NH_2 -terminus, the enzymically released fragment will have a free NH_2 -group at the N-terminus; the ϵ - NH_2 groups remain protected. By these reactions, one fragment can be prepared with only a C-terminal carboxyl group and another with only an N-terminal NH_2 group, appropriate to the coupling reaction. In this way, linking of the first peptide to the second can be made to take place specifically between the C-terminus of the one and the N-terminus of the other. While these reactions seem straightforward, it is true to say that few reports have appeared in the literature on the preparation of new polypeptides by the combination of natural fragments.

A semisynthesis of a different type has involved the preparation of an insulin in which the B1 phenylalanine was replaced by iodophenylalanine, with retention of biological activity (Offord, *Nature*, **227**, 718; 1970). To perform the conversion, the A1 and B29 NH_2 groups were protected by

trifluoroacetylation and the remaining NH_2 group at B1 was blocked by reaction with phenylisothiocyanate. The B1 phenylalanine residue was removed by treatment with trifluoroacetic acid, giving rise to a fragment of insulin which lacked only the B1 residue. Coupling of iodophenylalanine to the insulin fragment was easily achieved. Obviously the semisynthesis of this homogeneous derivative is more practicable than a total synthesis of the specifically iodinated insulin from the constituent amino acids.

Dyckes, Creighton and Sheppard (*Nature*, **247**, 202; 1974) have described an intriguing semisynthesis of an analogue of trypsin inhibitor. The 58-residue polypeptide was cleaved specifically at a methionylarginine residue by cyanogen bromide, which converted the methionine residue to homoserine lactone and conferred weak acylating properties on the fragment. A peptide bond then formed spontaneously between the homoserine residue and the adjacent arginine residue, restoring

the intact polypeptide chain. The product, which carried a residue of homoserine in place of methionine, retained the inhibitory action of the natural molecule. Corradin and Harbury (*Biochem. biophys. Res. Commun.*, **61**, 1400; 1974) performed a similar experiment with cytochrome c, which has no stabilising disulphide bridges. They found that cyanogen bromide cleavage gave rise to two fragments which could reunite; again the homoserine analogue retained biological activity.

In the general case, cleavage of a polypeptide at methionine would not be accompanied by a tendency for the fragments to recombine. Only in situations where the three-dimensional structure of the cleaved molecule is the same or very similar to that of the natural molecule will the newly released termini be held in juxtaposition for intramolecular condensation.

These are early days in the development of semisynthetic procedures. Future applications will be awaited with interest. $\square$

Between molecules and collision complexes

from our Chemical Physics Correspondent

It is almost three years (*Nature*, **240**, 257; 1972) since I drew attention to the identification of $(\text{HF})_2$ and expressed the hope that H_2OHF would also be identified in the gas phase. This has now been done by Bevan, Legon, Millen and Rogers (*J. Chem. Soc. Commun.*, page 341; 1975) who find a clear-cut microwave spectrum near 14 GHz and 29 GHz.

It is the hydrogen of the HF which forms the hydrogen bond; the O . . . F distance is 268 pm and the dipole moment is 12.8×10^{-30} C m (3.82 debye). There is an intensity alternation associated with the spin statistics of the two equivalent H attached to the oxygen, but it is not clear if the whole molecule is planar (point group C_{2v}) or whether the fluorine lies out of the H_2O plane (point group C_s). As expected the intensity seems to be proportional to the product of the partial pressures of each component and with an optimum total pressure of 100 Pa the lines are necessarily broad and such clear identification must have required the use of much experimental skill.

But though hydrogen bonds have been known for some time and such gas phase molecules have long been postulated, the extension of this work to the charge transfer species $(\text{CH}_3)_3\text{N} \cdot \text{ICF}_3$ is even more remark-

able (Legon, Millen and Rogers, *J. Chem. Soc. Chem. Commun.*, page 580; 1975) even though it is based on earlier infrared evidence (Mishra and Pullin, *Aust. J. Chem.*, **24**, 2493; 1971). For this species, which is a symmetric top, lines for J from 30 to 37 span 26 GHz to 33 GHz and are identified. With only one value of B for such a large molecule the structural information is not very precise, but suggests an N . . . I distance of 293.2 pm. Habit sets the expression 'N . . . I' with dots suggesting a non-bonded distance, but really the charge transfer complex has now a clear-cut identity and perhaps a full bond, based on charge transfer, should be shown.

And also unexpected is the spectroscopic detection, this time with molecular beam apparatus, by Harris, Janda, Novick and Klemperer (*J. Chem. Phys.*, **63**, 881; 1975), of the rare gas species Ar.OCS. Both radiofrequency and microwave transitions were seen and they establish the structure as basically T-shaped with the argon adjacent to the carbon at a distance of 358 pm, slightly greater than the van der Waals' radius sum.

All of these molecules with self-respecting structures, lifetimes and dipole moments fill in the gap between true molecules and sticky collision complexes.

review article

Ion implantation

G. Dearnaley*

Ion implantation has proved an elegant and successful technique, and in the manufacture of silicon devices, is now being extended both for research and practical applications in other materials, such as metals.

ION implantation is the process whereby controlled amounts of chosen foreign species can be introduced into the near-surface regions of a material in the form of an accelerated beam of ions. The energies used normally lie between 10 keV and 500 keV, with corresponding penetrations ranging from 100 Å to 1 µm, depending also on the target material.

The technique has proved highly successful for the fabrication of semiconductor devices, in spite of being in competition with well established processes of thermal diffusion or epitaxy, and requiring a relatively expensive accelerator and vacuum equipment. Some 200 accelerators are now in operation throughout the world and this number is expected to rise 10-fold—far in excess of the number used for nuclear research.

There are several reasons for the success of ion implantation. Among the most important are the control and resolution offered by this technique. Both the total amount and the purity of the implanted material can be accurately controlled and monitored, in a way that is impossible, for example, with diffusion, in which surface phenomena determine the dose which diffuses in. Furthermore, the concentration of impurities as a function of depth can be controlled by means of the ion energy, so that it is feasible to implant a buried layer of dopant. But for practical purposes, a crucial feature of the technique is its compatibility with photolithographic masking. This means that the well directed beam entering the surface produces a doped region which can be given a very high lateral resolution using conventional masking techniques (the etching of apertures through oxide films by use of a photosensitive resin overlayer).

The process also avoids certain problems associated with other techniques. Implantation, and the subsequent annealing of damage, are completed quickly and at relatively low temperatures, avoiding the difficulties associated with lengthy exposure to high temperatures; it is also very clean, being carried out in a vacuum, and could in principle be highly automated.

Its versatility is a further advantage, for a single accelerator can be used for many doping and other processes in device manufacture, with a schedule that is easily varied in such a way as to aid product development or the production of short runs of specialised devices. In materials other than semiconductors, the advantage lies in the possibility of introducing essentially any required species and producing a treated layer which is integral with the substrate.

Physical processes

When ions enter a solid, they lose energy through elastic collisions and through electron excitations. A reasonably accurate theory of the process was developed by Lindhard,

Scharff and Schiøtt¹, and this is widely used to calculate ion ranges and range distributions. Some results for silicon are shown in Fig. 1, from which it can be seen that the ranges of commonly used dopant ions (boron, phosphorus) are compatible with the dimensions of present-day microelectronic components (100–1,000 nm) with ion energies of a few hundred keV. Since the semiconductors are crystalline, the phenomenon of ion channelling² introduces some difficulties: this is the enhanced penetration of ions travelling along open crystallographic directions, as a result of small-angle collisions with the atomic rows. We have been able to show that this is the cause of a “tail” which extends beyond the expected maximum range (Fig. 2) and various techniques have had to be developed to cope with this.

Most semiconductors are covalently-bonded materials, with rigidly defined bond lengths and bond angles. Under ion bombardment this structure is vulnerable to disordering and in most cases it is necessary to restore crystallinity by thermal annealing at 800–1,200 K for about 20 min. Otherwise, electrically-active defects will dominate the effects of the atoms introduced. Figure 3 shows a transmission electron micrograph of a wafer of phosphorus-implanted silicon after annealing at 700 °C. There is a large amount of localised strain visible as dark regions, but the superimposed electron diffraction pattern shows that between these the crystal is relatively perfect. Fortunately, the electron transport properties of the semiconductor are also very good and the carrier mobility in the implanted zone may approach that of bulk material at the same impurity concentration. Moreover, the carrier density will (in silicon) correspond to the number of phosphorus or arsenic atoms introduced, although for boron the degree of electrical activity is commonly less than 100% because of the formation of complexes such as SiB₆. In some applications, the electrical effects of defects induced by bombardment can be put to good use. Proton bombardment will raise the resistivity of GaAs by seven orders of magnitude³, and is increasingly adopted for producing the isolation between neighbouring devices. The physical mechanisms involved are only just becoming clear: once again technology has provided a spur to physics. Another important process is that of causing unwanted metallic impurities, such as Au, Cu, Ni, to diffuse to defects introduced by ion bombardment⁴. This is the technique known as “gettering”, important for achieving reproducible device performance and low diode leakage currents.

Ion-implanted silicon devices

We can now consider the various semiconductor devices to which ion implantation has successfully been applied, illustrating the advantages I have mentioned. The first important structures

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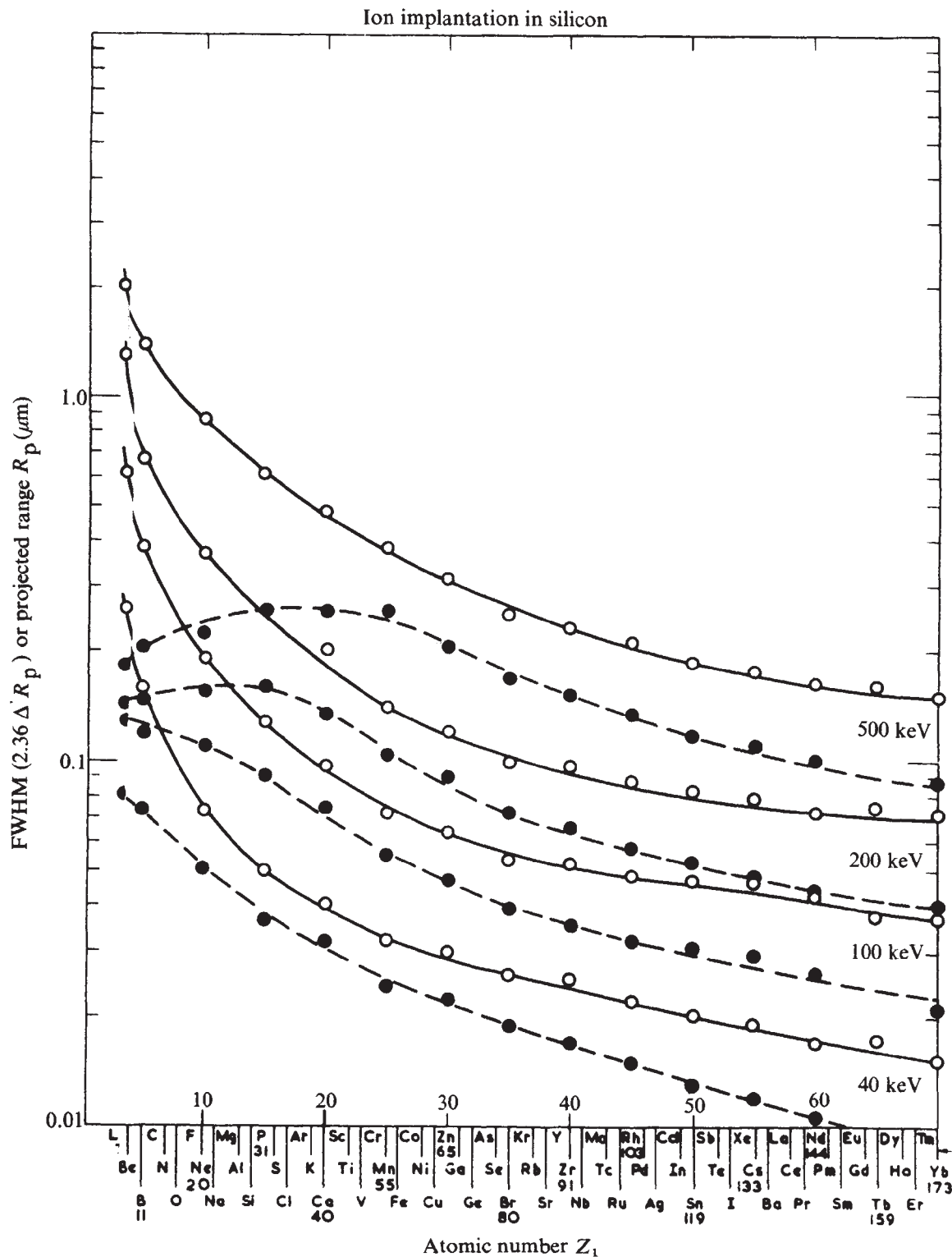


Fig. 1 Ion ranges in silicon, as a function of atomic number, and the full-width at half maximum (FWHM) of the depth distribution, assumed to be Gaussian. ○, Projected range; ●, FWHM (projected).

to benefit dramatically from the technique were the metal-oxide-semiconductor (MOS) transistors. In these the conduction takes place at the surface of a silicon crystal, and is controlled by the voltage applied to a metal "gate" electrode, electrically insulated from the silicon beneath by a thin layer of thermally-grown SiO_2 . Difficulties in aligning the elements of this structure were overcome by using the metal gate as a mask for implantation. Excellent registration of the "source" and "drain" (input and output) regions of the transistor was

automatically achieved with a consequent reduction in stray capacitance. Next, the surface conducting region was doped by ion implantation to the required value, while the material surrounding the transistor was maintained at a higher resistivity. By this means parasitic capacitances that load the input and output were significantly reduced. Finally, it was shown that the conducting "channel" could readily be buried so that electrons or holes would travel in good crystalline material rather than at the silicon-oxide interface where lattice defects

would interrupt their motion. These factors allowed the dimensions of MOS transistors to be reduced and their speed of operation to be dramatically improved.

Integrated circuits, sometimes containing many thousand individual transistors on a single square of silicon, have become extremely important for electronic systems ranging from pocket calculators to military defence installations, and they were stimulated intensively during the development of space satellites. Besides the techniques described above, two other applications of ion implantation have been found which proved even more beneficial. The threshold voltage, at which conduction of an MOS transistor commences, is governed by impurities in the oxide layer or at the oxide-silicon interface. Bombardment with low doses of ions (not necessarily group III or group V dopants) alters the threshold voltage⁶ in a remarkably controllable manner (Fig. 4). Secondly, it is desirable to construct the two main types of MOS transistor (called "enhancement" and "depletion" mode devices) within the same circuit, so that one can act as a load for the other. The result is a considerable saving in power consumption, important in pocket calculators or electronic wristwatches, and so on. The necessary doping, to produce p-type regions, or "wells",

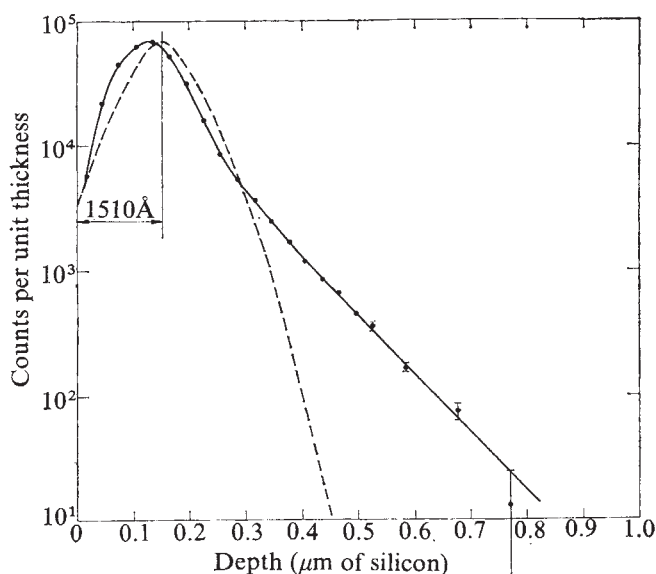


Fig. 2 Penetrating "tail" observed in the distribution of radioactive phosphorus ions implanted into a silicon crystal in a direction well removed from major channelling axes or planes. This tail is due to scattering into channelling directions. • $\gamma 13$ -X. 7° to $\langle 11 \rangle$. 120 keV. $5 \times 10^{12} \text{ cm}^{-2}$. ^{32}P . Room temperature. (---) calculated from Lindhard *et al.*¹.

in n-type silicon for these circuits is best carried out by implantation. In the most recent development both enhancement and depletion mode devices are implanted, and computer memories with almost 19,000 transistors on a single silicon "chip" have been produced. A microprocessor with the general capability of a PDP-11 computer is expected to be introduced this year, packed on to a chip which is comparable in size with the first calculator microcircuits.

Even these achievements are overshadowed by the rapidly developing "charge-coupled devices" in which, by an extension of MOS technology, arrays of metal-oxide-silicon capacitors are used to store charge which can be transferred to the adjoining storage element, just as in a solid-state analogue of the obsolete "Dekatron" glow-discharge indicator tubes. The charges can be liberated optically, in which case the device acts as a scannable photo-detector array, already on the market in the form of hand-held TV cameras. Alternatively, the devices can be used to store and process analogue information. It is important,

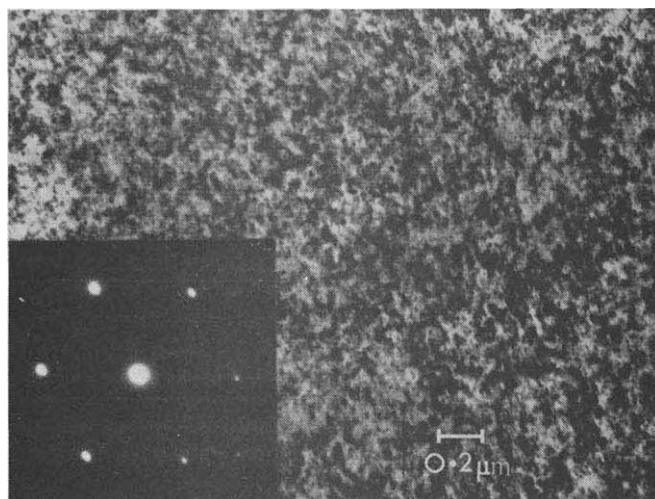


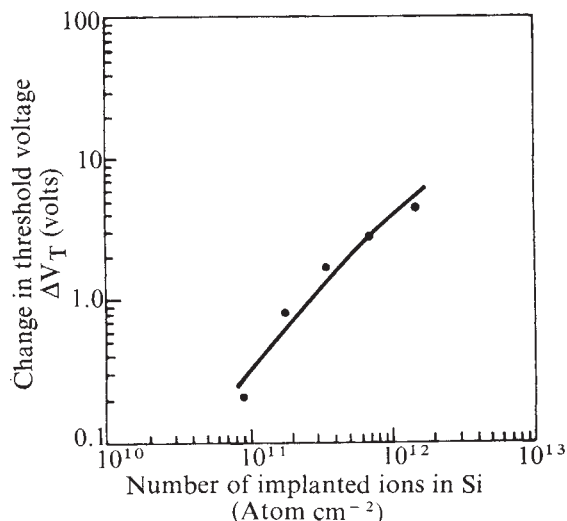
Fig. 3 Transmission electron micrograph of an ion-implanted silicon crystal, after thermal annealing, together with (inset) an electron diffraction pattern from the same region. The former shows a dense network of defects, while the latter reveals that the intervening matrix is crystalline.

however, that charge be transferred efficiently from one element to the next, and here the "buried channel" produced by ion implantation has proved highly effective, removing the charges from the region containing traps at the oxide interface. It seems likely that ion bombardment gettering will be useful for removing metallic impurities which trap mobile carriers.

Bipolar transistors, in which the active elements (emitter, base and collector) are buried within a silicon crystal, still account for the major part of the device market. At first it seemed that they would not benefit from ion implantation, since the performance of implanted transistors was not reproducible. It is now realised that this was due, in part, to the effects of the "tails" on implanted ion distributions (Fig. 2), which vary in extent from one crystal to the next and which influence the depth of emitter-base junctions. It was found in the USA (R. J. Scavuzzo, R. S. Payne, K. H. Olson, J. M. Nacci and R. A. Moline, unpublished paper presented at the IEEE Electronic Devices Meeting, Washington DC, 1972) that if arsenic ions are implanted to form the emitter a subsequent "drive-in" diffusion will yield a desirable concentration profile

Fig. 4 An example of threshold control in an MOS transistor achieved by boron ion implantation (after Sigmon and Swanson⁶). 30 keV implant oxide thickness $\approx 550 \text{ Å}$ boron, 500°C anneal.

● Experimental.



with a reproducible distribution of electrically active donors. Other improvements have come from a better control of the size of residual defects (Fig. 3) which remain after annealing, and which can, if large, project through the junction with undesirable local effects on conduction. By a careful choice of annealing schedule these defects can be reduced to a negligible 10 nm or so in size. Excellent wholly implanted bipolar transistors have thus been produced in many laboratories, and this has led to a considerable upsurge in interest in ion implantation, which is now seen as an important process for practically all new device fabrication.

Specialised diodes, such as the variable-capacitance devices used for tuning purposes in radio and TV, or avalanche photodiodes which are fast, sensitive optical detectors, or nuclear particle detector diodes are all made more controllably by ion implantation. Indeed, yield improvements of two orders of magnitude have been reported for variable-capacitance diodes. However, these structures account for only a small fraction of silicon device output. A more promising development is in high-power rectifiers, in which ion implantation provides a close control of doping within a very thin layer. This thin layer can store very few trapped charges and hence a fast recovery is achieved without the need for the usual gold doping. 100-A, 150-V ion-implanted rectifiers are already on the market, and it is forecast that the process will be useful for diodes operating at up to 3,000 A.

Compound semiconductor devices

At first it was widely believed that ion implantation would speedily advance the fabrication of compound semiconductor devices, because it is in these materials that thermal diffusion is least satisfactory. Unfortunately this has not come about, for several reasons. In compounds, ion bombardment can create a wider variety of defects in which, for example, atoms are located on the wrong lattice sites. Some of these defects, after clustering, are thermally very stable, and the compound becomes unstable at temperatures below that required to restore order.

The problem then becomes one of finding a suitable encapsulating material which will prevent loss of the more volatile constituent during annealing. In GaAs (the most widely-used compound semiconductor) it has not proved easy to find a practicable means of encapsulation, but progress is being made along several ingenious routes. A good deal of exploration has been carried out to find the most suitable dopants, and Si^{4+} ions have been shown to provide a high degree of donor activity⁷.

More immediate success was achieved in the inverse direction, that is that of destroying electrical conductivity in selected areas of semiconductor by ion bombardment. Modest doses

(10^{14} – 10^{15} cm^{-2}) of protons will render GaAs, GaP or InP semi-insulating, in a manner that is very useful for isolating one device from its neighbour. In GaP, for example, the bombardment also turns the material opaque, and this has been exploited in a light-emitting diode array in which the individual elements are isolated both optically and electrically (A. R. Peaker, unpublished paper delivered at the European Solidstate Device Research Conference, Nottingham, 1974). Proton-induced defects are not very stable, however, since they probably consist of very small disordered regions and they are rather easily annealed out. There is a need for a less empirical approach to this useful process.

Equipment for ion implantation

The design and manufacture of ion accelerators and target chambers for ion implantation, in research and production, has become a sizeable industry, with an estimated world market of over £100 million over the next five years.

Some of the more useful ion sources for implantation purposes were developed originally for the separation of isotopes, where a simple, rugged source capable of delivering intense currents of a variety of ion species is also required. For research purposes it is advantageous to be able to change from one ion beam to another without elaborate clean-up procedures, while for industrial applications the reliable and efficient production of a few important ion species is paramount.

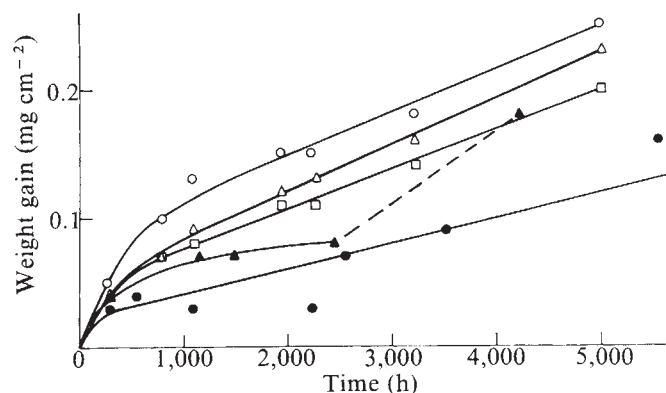
There are two possible configurations for the acceleration system: in one case ions are mass-analysed after reaching their full energy while in the other magnetic analysis is carried out after acceleration through only 20–40 keV and the selected beam is further accelerated to the required energy. The latter has the advantages that the high-voltage supply need not be well stabilised, and only the specific ions required are accelerated so that X rays resulting from back-streaming secondary electrons are minimised. Either the magnetic analysis system or the target chamber must be at a high potential, however—a feature which can pose a few design problems. Both these arrangements have been adopted in systems based on the Lintott accelerator. This equipment delivers the highest currents of any commercial machine, and consideration has had to be given to the consequences of target heating since a 1 mA beam at 100 keV dissipates 100 W and this power is not readily conducted from semiconductor wafers in vacuum. The solution⁸ has been to manipulate many wafers rapidly through the beam, so spreading the heat dissipation, while also achieving a high degree of uniformity in the dose (ions per unit area). In this way, a throughput of several hundred 5 cm-diameter silicon wafers can be achieved in a day, and the process becomes very economical. In most research facilities, however, the ion beam is swept electrostatically over the face of a single fixed specimen, but it is not easy to achieve such a high degree of uniformity in dose. For example, neutral atoms resulting from collisions with residual gas are undeflected and give a central spot in the distribution.

Accurate current measurement, in the presence of secondary electrons and secondary ions, is not a trivial matter and comparisons between results obtained on different implantation systems can be surprising. Work is in progress to develop an absolute calorimetric ion collector, and to provide means of calibrating accurately an implanted specimen against a laboratory standard, using an ion backscattering technique at MeV energies.

Ion implantation of metals

During the past year or two it has become clear that ion implantation has important applications outside the semiconductor field, and an investigation has begun of the effects of implantation upon the surface properties of metals and alloys. The corrosion behaviour, electrochemical properties, coefficient of friction, wear resistance and bonding ability of metals are controlled by their near-surface composition. Novel

Fig. 5 The effect of yttrium implantation in a niobium-containing steel alloy, compared with that of alloyed yttrium, on the oxidation of the metal in CO_2 at 700 °C (after Antill *et al.*¹¹). ○, 20/25/Nb; □, 20/25/Nb 0.13% Y alloy; △, 20/25/Nb 0.41% Y alloy; ●▲, Y implanted 20/25/Nb.



experiments on all these phenomena can be made by exploiting the ability to alter the surface composition in a versatile manner, and to monitor the migration of atoms initially confined to a shallow layer.

Thus at Harwell, we have studied the effects on thermal oxidation as a result of the implantation of a wide variety of ion species into titanium, zirconium and stainless steel⁹. The results in titanium and steel indicate a close correlation between the electronegativity of the impurity ions and the oxidation rate, but effects were reversed in the two metals. The behaviour of zirconium was quite different from that of titanium in spite of their proximity in the Periodic Table. There was no correlation with electrochemical properties but some indications of a trend with ionic size, which has been observed also in alloy studies. We inferred that these striking differences arise from the crystallographic properties of TiO₂ and ZrO₂. Crystallographic shear in TiO₂ is a powerful mechanism for eliminating oxygen vacancies, while in ZrO₂ the mechanical stress caused by volume changes upon oxidation is relieved by cracking and pore formation. The ionic size of impurities will influence this stress, and thus the degree of oxygen transport along fissures.

In these metals, bombardment by inert ions, or ions of the same species as the target produced little or no change in oxidation rate. But in the case of metals such as chromium or nickel, cation out-diffusion is the dominant process during oxidation and this seems to be inhibited by Cr⁺ or Ni⁺ bombardment, respectively.

This effect, together with the observation by Hartley¹⁰ of a striking increase in wear resistance of ion-implanted steels, can tentatively be explained as a result of the lateral compressive stress generated by ion implantation. Such stresses can approach the yield stress of the material, and thus have a considerable influence on grain boundary phenomena and surface plasticity.

Besides these scientific studies, the practical possibility of

treating metallic components by ion implantation to improve their surface properties is arousing interest. Antill and others at Harwell have demonstrated¹¹ that implanted yttrium can be as effective as alloyed yttrium for inhibiting the high-temperature oxidation of an austenitic stainless steel in CO₂, without the drawbacks of a reduced bulk tensile strength and ductility. In this and other cases still under investigation the duration of the effects of ion implantation is remarkably long. This is due, in the case mentioned, to the fact that the yttrium remains near the metal-oxide interface. In other instances, and in wear modification, it could be that the initial phase of the corrosion or wear process is crucial in determining the subsequent pattern of behaviour.

In such practical applications the successful exploitation of ion implantation will depend upon economic factors, the geometry of the workpiece and the merits of alternative techniques. Novel configurations of ion accelerator and more versatile designs of ion sources will need to be developed, but the costs of the process seem likely to be acceptable for a variety of applications in both high and low cost systems.

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articles

Principles of protein-protein recognition

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The formation of the protein-protein interface by the insulin dimer, the trypsin-PTI complex and the αβ oxyhaemoglobin dimer removes 1,130–1,720 Å² of accessible surface from contact with water. The residues forming the interface are close packed: each occupies the same volume as it does in crystals of amino acids. These results indicate that hydrophobicity is the major factor stabilising protein-protein association, while complementarity plays a selective role in deciding which proteins may associate.

BIOLOGICAL molecules are able to recognise each other and form specific associations. These processes are fundamental

to biology and the nature of the interactions involved has frequently been discussed.

Recently X-ray diffraction analysis has provided detailed atomic information on several systems which involve protein-protein recognition and association. As a result of this we can now approach directly the problem of the nature of the interactions. We shall use here the known atomic structures of dimeric porcine insulin¹, of the bovine trypsin-pancreatic inhibitor complex^{2,3} and of the αβ dimer of horse oxyhaemoglobin⁴.

The association between protein subunits in these systems has been described in terms of Van der Waals' contacts, of electrostatic forces and of hydrogen bonds: that is, of point-to-point interactions. Here we analyse the contact regions between subunits in terms of surface area and of the volumes

occupied by residues, and show how these quantities, which can be interpreted from a thermodynamic point of view, account for the specificity and the stability of the associations.

Area and chemical nature of surfaces involved in protein-protein association

The concept of accessible surface area⁵ describes the extent to which protein atoms can form contacts with the solvent. For a given atom it is defined as the area over which the centre of a water molecule can be placed while retaining Van der Waals' contact with that atom and not penetrating any other atom. Accessible surface areas are correlated with hydrophobic free energies⁶.

Using available atomic coordinates and a computer program written by M. Levitt, we have calculated in the manner described previously⁷, the total area accessible to water for each protein complex and for the isolated subunits. The surface area buried in the complex is then defined as the accessible surface area of one subunit plus that of the other subunit minus that of the complex (Table 1). This assumes that the free subunits have the same conformation as found in the complex. For the pancreatic trypsin inhibitor (PTI), the structure of which has been determined independently as the free molecule⁸ and as the trypsin-PTI complex², we find that the small variations of conformation which do occur on binding have little effect on the estimation of the surface areas (Table 1).

Table 1 Changes in accessible surface area

Complex	Total accessible surface area (Å ²)	Area buried in complex (Å ²)	No. of residues involved*
Insulin dimer			
Monomer M1	3,400	530	16
Monomer M2	3,310	600	18
Total change		1,130	14
Trypsin-PTI			
Trypsin	8,920	640	7
PTI	3,700	750†	20
Total change		1,390	12
Horse oxyhaemoglobin			
α subunit	6,940	930	13
β subunit	7,130	790	11
Total change in α β		1,720	

*Number of residues contributing more than 10 Å² to the surface area buried in the complex. In all cases residues which contribute less than 10 Å² make up for 2-7% of the total buried area.

†Value obtained as described in text. A value of 815 Å² was calculated using atomic coordinates for free PTI rather than the PTI moiety of the complex.

For each of the three interactions studied here, the association of the protein subunits leads to a reduction of the surface area accessible to solvent by more than 1,000 Å². The chemical nature of the groups involved is analysed in Table 2, which details the relative contributions of polar and non-polar atoms to the accessible surface areas of the molecules and to the interfaces. Eight aromatic residues occur in the insulin dimer interface (Tyr B16, Phe B24, Phe B25 and Tyr B26, from each monomer) making that interface a good deal more hydrophobic than the rest of the protein surface. This is less true of the αβ interface in haemoglobin, while the trypsin-PTI interface has essentially the same chemical composition as other parts of the protein surface which remain accessible to the solvent. Thus, a number of polar or charged atoms become inaccessible to solvent when the complexes form: Table 3 shows that most, if not all, form hydrogen bonds.

Protein interfaces are close packed

Richards⁹ has shown that the volume occupied by an atom inside a protein molecule could be defined by tracing an

Table 2 Chemical character of protein surface

Surface	Contribution to surface areas of non-polar* (%)	polar† (%)	charged atoms (%)
Insulin			
Monomer M1	62	31	7
Monomer M2	59	33	8
Interface	74	22	4
Trypsin-PTI			
Trypsin	53	38	8
PTI	57	23	20
Interface	56	32	12
Oxyhaemoglobin			
α subunit	64	20	16
β subunit	58	23	19
Interface	68	16	16

*All C atoms.

†N, O and S atoms except in charged groups.

appropriate space-filling polyhedron (Voronoi polyhedron) separating that atom from adjacent atoms. This method was used to demonstrate that protein interiors are close packed⁹ with individual amino acid residues occupying the same volume as they do in crystals of the amino acids themselves⁷. Using Richards' computer program in the manner described previously^{9,7}, we have calculated the volume occupied by residues buried in the interfaces: that is, by residues which are inaccessible to solvent in the complex but have a large accessible surface area in the isolated subunits. The results are summarised in Table 4, which also lists the volume occupied by residues in protein interiors and in crystals of amino acids. Table 4 shows clearly that residues occupy the same volume in protein interfaces, inside a protein or in the crystalline amino acid. The standard deviations are ~ 6% and a loose packing would cause a systematic tendency of the volumes towards higher values. The total volume of the residues cited in Table 4 for each complex, however, is in all cases within 1% of the corresponding volume calculated from crystalline amino acids. Thus the same rule applies to small organic molecules in the crystal state, and to amino acid residues in protein interfaces (or inside proteins): they close pack.

Free energy of protein association

The free energy of dissociation ΔG_D of a complex between two molecules is related to its dissociation constant K_D by:

$$\Delta G_D = -RT \ln K_D \quad (1)$$

This equation implicitly refers to a standard state where all chemical species are 1 M, and attributes a free energy of zero to a complex with a dissociation constant of 1 M.

When two molecules associate, there is a loss of translational and rotational entropy which can easily be derived from

Table 3 Buried polar atoms and hydrogen bonds

Complex	No. of polar atoms buried in interface	Atoms buried forming H bonds	No. of new H bonds formed*
Insulin dimer	12	12	4†
Trypsin-PTI	29	25‡	13
Haemoglobin α β dimer	31	27§	10
Total	72	64 (89%)	

*Requirements for H bonds include proper orientation of groups and a maximum distance of 3.7 Å (in fact all but four are within 3.3 Å).

†These bonds have been described in ref. 1.

‡Exceptions are: Sγ Cys 42 of trypsin, Sγ Cys 14, O Ala 16 and O Arg 17 on PTI.

§Possible exceptions are: Nε His α103, Nδ and Nε His α122, and Sδ Met β55. More recent data (R. Ladner, personal communication) suggest that the first three are in fact hydrogen bonded.

statistical thermodynamics (refs 10 and 11 and our unpublished work) at least in the case of perfect gases. While this might appear quite irrelevant to macromolecules in aqueous solutions, Page and Jencks¹¹ have shown that the calculation gives reasonable estimates of the entropy lost by small organic molecules reacting in solution. Applying it now to the complexes studied here, we find that the entropy corresponding to three translational and three rotational degrees of freedom lost when two molecules associate amounts to 70–100 cal deg⁻¹ mol⁻¹, equivalent to a free energy, ΔG^s_D , of 20 to 30 kcal mol⁻¹ at 27 °C (in the standard state). To form a complex with a

(ref. 19), the difference between a protein–protein H bond and the corresponding bond made with water in the dissociated complex can only be a fraction of this. It is unlikely that the four H bonds of the insulin dimer or even the 13 H bonds of the trypsin–PTI complex contribute more than 4–15 kcal mol⁻¹ to the dissociation free energies.

Van der Waals' interactions are both much less energetic²⁰ and much more numerous than H bonds as they involve all pairs of neighbouring atoms. But as atoms making Van der Waals' interactions in the complex make similar interactions with water in the dissociated subunits, the overall contribution is small. Still, like hydrogen bonds, Van der Waals' interactions place severe restrictions on the structure of protein surfaces: to achieve the same interactions with each other as they do with water, the two surfaces must be complementary, and this is expressed in the close packing of residues, which we have described.

Hydrophobic contribution

We have ignored so far the effect of solvent entropy on the thermodynamic balance of the association: that is, the hydrophobic contribution. For this, we shall use an empirical correlation found between the accessible surface area of a solute and its free energy of transfer to a water solution. Such a correlation has been established for hydrocarbons and amphiphilic molecules^{21,22}, and also for amino acids⁶: for the latter, 1 Å² of surface area corresponds to 25 cal mol⁻¹ of hydrophobic free energy⁷.

A free energy term favouring association is thus derived from the decrease in surface area accessible to water occurring when proteins associate. Using the values of Table 1, we calculate the hydrophobic contribution to the formation of the insulin dimer, of the PTI–trypsin complex and of the $\alpha\beta$ dimer of haemoglobin. Table 5 shows that this contribution is very large in each case, and of the same order as the value of ΔG^t_D estimated above: in other words, the entropy gained by water due to the smaller accessible protein surface area is sufficient to compensate the entropy lost by protein molecules forming a complex.

Stability and complementarity

These results show that hydrophobicity is the major factor which stabilises protein–protein associations. Our estimates of the free energy required to make a stable complex indicate that each subunit needs to bury about 600 Å², corresponding to a hydrophobic contribution of 30 kcal mol⁻¹ (for two subunits). In the case of the trypsin inhibitors this surface

Table 4 Volume of residues buried in subunit interfaces

Residue	No.	Volume			
		V_s^*	σ of V_s	$V_{P\dagger}$	$V_{H\dagger}$
Gly	7	68.5	6.6	66.4	66.5
Phe	6	196.3	7.8	203.4	—
Ala	5	89.4	6.9	91.5	96.6
Val	5	141.1	6.7	141.7	143.4
Pro	5	128.9	11.7	129.3	124.4
Leu	2	161.3	9.4	167.9	—
Ile	1	175.2	—	168.8	169.7
Ser	4	99.7	7.0	99.1	102.2
Tyr	3	205.1	9.6	203.6	201.7
Cys	2	108.9	1.7	105.6	108.7
Trp	1	241.6	—	237.6	—
His	4	167.6	13.5	167.3	166.3
Gln	4	165.5	7.0	161.1	148.0
Asn	1	147.4	—	135.2	—
Arg	3	202.1	3.2	—	—
Lys	2	172.9	1.3	171.3	—
Asp	1	127.2	3.7	124.5	122.0

*Average volume of residue in protein interfaces.

All residues contributing more than 10 Å² to the buried surface area and having less than 20% of their surface accessible in the complex are included.

†The average volume of the residue when buried in the protein interior⁷.

‡The volume of the residue when in crystals of the amino acid⁷.

dissociation constant K_D , one must therefore provide a free energy at least equal to:

$$\Delta G^t_D = \Delta G^s_D + \Delta G_D \quad (2)$$

Table 5 shows that weak but observable protein–protein interactions such as found in the insulin dimer require at least 25–35 kcal mol⁻¹ of free energy, while the tight binding of PTI to trypsin or of the α and β subunits in haemoglobin needs 30–50 kcal mol⁻¹.

Origin of free energy of association

Can the structural analysis of the protein interfaces described above account for these large energies? What relative contribution does each type of interaction make to the association? Pauling¹⁵ has argued that energy of association by proteins is due to the complementarity in structure and the resultant cooperation of weak forces (that is, H bonds and Van der Waals' interactions) over a sufficiently large area to produce a strong bond. An alternative model, due to Kauzmann¹⁶, stresses the importance of hydrophobic energies, the association areas of proteins being viewed as surface patches of non-polar side chains brought together rather like hydrocarbon chains in lipid micelles.

Hydrogen bonds and Van der Waals' interactions

Thermodynamic evidence is against hydrogen bonds making a major contribution to the free energy of protein–protein interaction^{17,18}. Although the enthalpy of breaking a typical amide H bond may be of the order of 4–5 kcal mol⁻¹

Table 5 Free energy of protein–protein association

Complex	Insulin dimer	Trypsin–PTI	Haemoglobin $\alpha\beta$ dimer
Dissociation constant K_D (molar)†	10 ⁻⁵	10 ⁻¹³	≤10 ⁻⁸
Free energy of dissociation ΔG_D (kcal mol ⁻¹)†	7	18	>11
Translational/rotational free energy‡ ΔG^s_D (kcal mol ⁻¹)	23	27	27
Free energy required for association ΔG^t_D (kcal mol ⁻¹)§	30	45	>38
Surface area buried in the complex (Å ²)	1,130	1,390	1,720
Hydrophobic free energy (kcal mol ⁻¹)¶	28	35	43

*Data at neutral pH near 27 °C from refs 12–14.

† $\Delta G_D = -kT \ln K_D$.

‡Calculated from the translational/rotational partition functions.

§ $\Delta G^t_D = \Delta G^s_D + \Delta G_D$.

¶25 cal mol⁻¹ per Å² of buried surface area.

area is derived from only a small number of amino acid residues (ref. 3 and our unpublished work). As the hydrophobic contribution is entirely unspecific, it would lead to all kinds of incorrect interactions in a cell. The proper formation of hydrogen bonds and of Van der Waals' contacts, however, requires complementarity of the surfaces involved: they must be able to close-pack together, with the polar atoms properly positioned to make hydrogen bonds. Thus while Van der Waals' and polar interactions contribute little to the stability of the complex, they decide which proteins may recognise each other. Incorrect associations are forbidden by large unfavourable enthalpies due to poor packing and loss of hydrogen bonds made to water. These principles are likely to be also true of the interactions of proteins with small ligands, nucleic acids, and membranes.

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Chloroplast DNA from higher plants replicates by both the Cairns and the rolling circle mechanism

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The chloroplast DNA (ctDNA) from pea and corn plants contains both Cairns type and rolling circle replicative intermediates. Denaturation mapping studies with pea ctDNA molecules have shown that the rolling circles initiate replication at or near the site where the Cairns replicative intermediates terminate replication. These results suggest that the rolling circles are initiated by a Cairns round of replication. A model for the replication of the chloroplast DNA is based on these results.

The chloroplast DNAs from higher plants and the alga *Euglena* have been isolated as covalently closed circular molecules with molecular weights from 85×10^6 to 96×10^6 (refs 1–3). The replication of corn and pea ctDNA^{4,5} has been shown to initiate with the formation of two displacement loops (Fig. 1b) similar to the displacement loops that initiate animal mitochondrial DNA (mtDNA) replication^{6,7}. The two displacing strands are hydrogen bonded to the opposite parental DNA strands, they expand toward each other (Fig. 1c), and form a structure that looks like a Cairns replicative intermediate when the two displacing strands elongate past each other (Fig. 1d). We present here evidence that pea and corn ctDNA replicate by both the Cairns replication mechanism⁸ and by the rolling circle mechanism⁹. Electron microscopic examination of pea and corn ctDNA have shown the presence of Cairns type and rolling circle type structures in both ctDNAs. Denaturation mapping studies on pea ctDNA have shown that the Cairns replicative intermediates elongate bidirectionally. The de-

naturation mapping experiment revealed that all the rolling circles initiated replication at the same site but could elongate in either direction from that site. The initiation site for the rolling circle round of replication was found to lie at or near the termination site of the Cairns round of replication, suggesting that the rolling circle replicative intermediates are initiated by the Cairns round of replication.

Structure of intermediates

To obtain replicative intermediates, the pea ctDNA was fractionated by centrifugation in ethidium bromide-caesium chloride density gradients^{3,7}. The DNA was collected into three fractions; the lower band of closed circular DNA containing 20–25% of the total pea ctDNA, the middle band

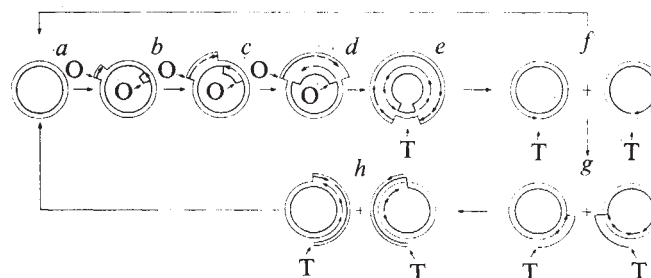


Fig. 1 A model for the replication of ctDNA. *a*, Closed circular parental molecule; *b*, D-loop containing molecule; *c*, expanded D-loop containing molecule; *d* and *e*, Cairns type of replicative intermediate; *f*, nicked progeny molecules; *g* and *h*, rolling circles. The thin and thick lines mark the opposite strands of a molecule. The lines with the arrows are the daughter strands. O indicates the positions of the two origins of D-loop synthesis which are 5.2% of pea ctDNA apart. T indicates the terminus of the Cairns round of replication which is 180° opposed to the origins of D-loop synthesis.

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containing 10% of the pea ctDNA, and the upper band of nicked circular and linear DNA containing 65–70% of the pea ctDNA (60% of the upper band DNA was circular). The upper band DNA was determined to be ctDNA by its buoyant density in CsCl and its renaturation rate³. These fractions were examined in the electron microscope using the formamide

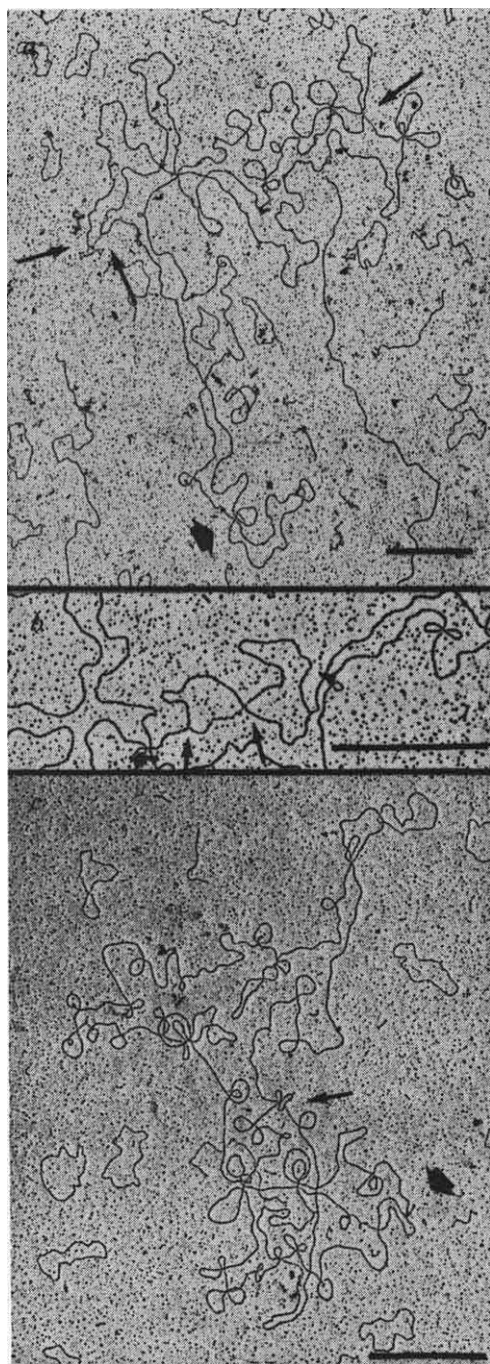


Fig. 2 Cairns replicative intermediates. *a*, A molecule that is 37% replicated and contains a single strand region (two small arrows) at one growing fork and a denatured region (large arrow) in the unreplicated portion of the molecule. *b*, A portion of a molecule that is 5.2% replicated and contains a single strand region (two small arrows) at one growing fork. *c*, A Cairns replicative forked molecule showing branch migration at one of the replicative forks (large arrow). The molecules were mounted for electron microscopy by the formamide technique^{10,18}; the spreading solution contained 50% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.5, the hypophase contained 20% formamide, 0.01 M Tris, 0.001 M EDTA, pH 8.5, and the spreading was performed at 23 °C. Single-stranded and double-stranded Φ X DNA molecules were used as internal standards in all experiments. The bars indicate 1 μ m.

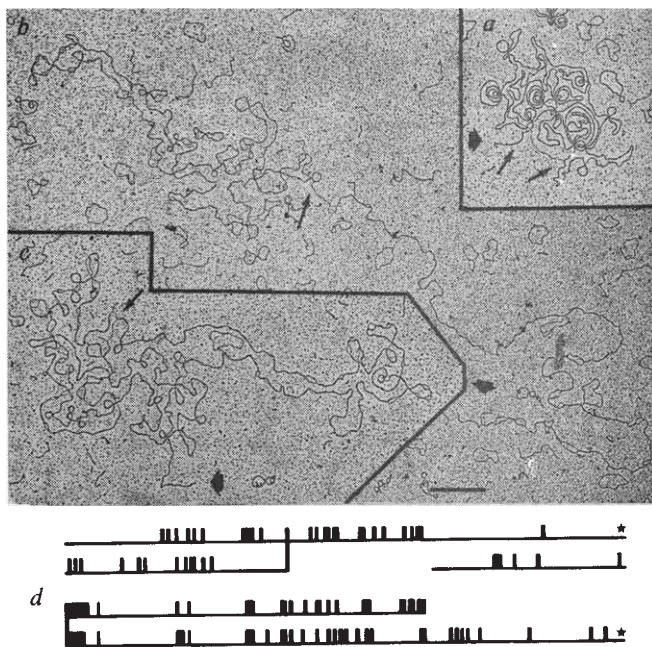


Fig. 3 Rolling circle molecules. *a*, A molecule with a tail that is 19% of the length of the attached circle. There is a single-strand region at the growing fork that is 3 500 bases long (indicated by arrows). This molecule was mounted for electron microscopy using the conditions described in Fig. 2 *b* and *c*. Partially denatured molecules with tails that illustrate the two different denatured patterns at the tip of the tails. These molecules were mounted for electron microscopy under partially denaturing conditions using the formamide technique as previously described¹⁸. The spreading solution contained 77% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.5, the hypophase contained 47% formamide, 0.01 M Tris, 0.001 M EDTA, pH 8.5, and spreadings were performed at 23 °C. Single-stranded and double-stranded Φ X DNA molecules were used as internal standards in all experiments. *a*, Graphical representation of molecules in *b* and *c*. The boxes indicate the denatured regions, the asterisk indicates the circular part of the molecule that has been linearised. The unmarked free end is the tip of each tail. The bars indicate 1 μ m.

technique to demonstrate single-stranded DNA¹⁰. Cairns type replicative forked structures were found in each fraction from two different DNA preparations (Fig. 2). Forty-five Cairns structures were measured and the extent of their replication ranged from 5.6 to 87%. Three per cent of the circular molecules in the lower band were Cairns structures and the extent of their replication ranged from 5.2 to 8.2%. The Cairns replicative intermediates made up an average of 5.7% of the circular pea ctDNA molecules in the middle band and the extent of their replication ranged from 7 to 50%. The Cairns structures consisted of 2.9% of the circular pea ctDNA molecules in the upper band and the extent of their replication ranged from 38% to 87%. In this study 3,118 circular pea ctDNA molecules were scored and it was calculated that 3.3% of the total circular pea ctDNA molecules consisted of Cairns replicative intermediates. The finding of Cairns replicative intermediates in the lower and middle bands of the caesium chloride–ethidium bromide density gradients, and the correlation between higher banding position and larger amounts of replication suggests that replication takes place on a covalently closed circular template, and is accompanied by nicking and closing cycles. Similar observations have been made in studies of the replication of mouse L cell mtDNA¹¹ and SV40 DNA¹².

The replicative intermediates of corn ctDNA were also studied. The total corn ctDNA was collected as a single fraction and 309 circular molecules were scored. Cairns replicative structures made up 4.5% of the total circular corn ctDNA molecules and the extent of their replication ranged from 9.4% to 70%. Cairns structures have also been found in *Euglena* ctDNA¹³.

In the above experiments, monomer-length circular molecules with an attached double-stranded tail were also observed (Fig. 3a). In the pea ctDNA preparation, circular molecules with tails accounted for 4.9% of the circular molecules in the upper band, and 2.8% of the circular molecules in the middle band. No circular molecules with tails were observed in the lower band of pea ctDNA (<0.05%). The circular molecules with double-stranded tails made up 3.1% of the total circular pea ctDNA. The lengths of tails in the pea ctDNA ranged from 1.5 to 124% of the length of the attached monomer-length circular molecule. Circular molecules with double-stranded tails were also found in the preparation of total corn ctDNA that was examined. In corn ctDNA, 11% of the total circular molecules had tails attached to them. The tails in the corn ctDNA ranged in length from 2% to 140% of the attached monomer length circular molecule. Fifteen per cent of all rolling circle forms had tails longer than the unit length of ctDNA. The finding of tails that were longer than the attached monomer length circular molecule eliminates the possibility that the tails arose by breakage of a Cairns forked structure at a replicative fork. This suggests that the circular molecules with tails are rolling circle replicative intermediates⁹.

Single-stranded regions in intermediates

A careful examination of the rolling circles and the Cairns forked structures in both pea and corn ctDNA showed that 77 to 81% of the growing forks in these molecules contained single-stranded regions (Figs 2a and b, and 3a). The lengths of these single-stranded regions were variable and ranged in length from 250 bases to 7,600 bases. The structure and frequency of these growing forks is presented in Fig. 4. In the Cairns structures, single-stranded regions could be observed at both of the replicative forks in a given molecule, which suggests that the Cairns structures grow bidirectionally. In unambiguously interpretable Cairns structures that contained single-stranded regions at both replicative forks, the single-stranded regions were located at positions that were *trans* with respect to each other (Fig. 4). In both the rolling circles and the Cairns structures, the single-stranded regions were located on only one daughter strand at each replicative fork. The single-stranded regions at the rolling circle growing forks were always found on the tail. These results indicate that at least one daughter strand at each growing fork is being synthesised discontinuously. The above observations do not eliminate the possibility that both daughter strands at each fork are being extended discontinuously, as in the case of T7 DNA¹⁴ or *E. coli* DNA¹⁵, since the failure to see gaps on both daughter strands of a growing fork would simply be a consequence of the 5' to 3' direction requirement of DNA synthesis¹⁶.

Short single-stranded tails were observed (Fig. 2c) at some of

Fig. 4 Diagrammatic representation of the growing forks. a and b, Cairns replicative forks; c, rolling circle replicative forks. The stars indicate the parental part of the Cairns replicative intermediates.

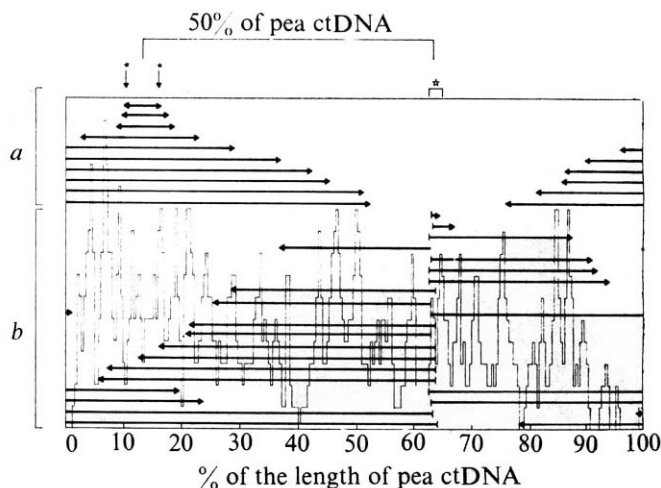
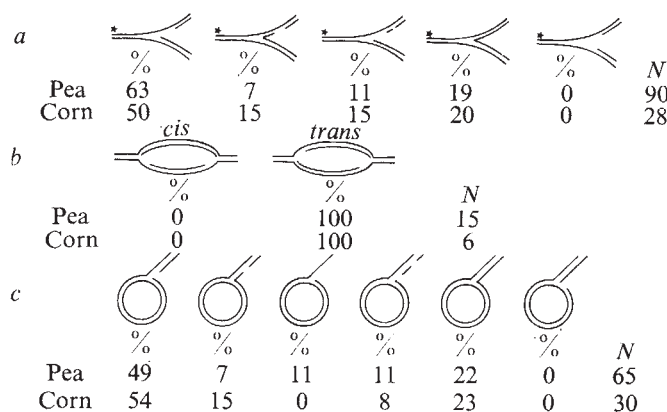


Fig. 5 Denaturation mapping of the replicative intermediates. DNA fractions containing replicative intermediates were mounted for electron microscopy under conditions of partial denaturation as described in the legend of Fig. 3. Under these conditions an average of 22% of each DNA molecule is denatured¹⁸. Three denaturation maps were constructed as described before¹⁸ from the clean circular ctDNA molecules, from the circular part of ctDNA molecules containing double-stranded tails, and from the Cairns type of forked molecules. All three denaturation maps were very similar showing that all the molecules examined had an identical sequence. An example of such a denaturation map is represented by the histogram in this figure. The orientation of the replicated portion of 10 Cairns replicative intermediates in this denaturation map is represented by the horizontal lines (a). The arrows at the ends of the lines indicate the positions of the replicative forks. The tails of the rolling circle molecules were oriented in the denaturation map by comparing the denaturation pattern of the tail with both the denaturation pattern of the attached monomer length circular molecule and the denaturation map essentially as described by Schnös and Inman¹⁹. The orientation of the tails from 20 rolling circles are represented by horizontal lines (b). The single arrow on each line indicates the position in the denaturation map where the tail was attached to the circular molecule and represents the position of the replicative fork. The other end of the horizontal line (b) marks the position in the denaturation map where the end of each rolling circle tail maps. The small stars indicate the two origins of D-loop synthesis and the larger star indicates the origin of the rolling circle synthesis.

the replicative forks in pea and corn ctDNA replicative intermediates. These whiskers have been suggested to arise from branch migration at the replicative fork¹⁷ and have been observed in the replicative intermediates from various systems¹⁵. Whiskers were observed at 17 and 15% of the rolling circle growing forks in pea and corn ctDNA, respectively. The Cairns structures in pea and corn ctDNA had whiskers at 13 and 15% of the growing forks, respectively. In addition to the whiskers, 12% of the pea ctDNA Cairns replicative intermediates found in the middle and lower bands had denatured regions in the unreplicated portion of the molecule (Fig. 2a). The relationship between branch migration and denatured regions in covalently closed replicative intermediates has been discussed elsewhere⁷.

Denaturation mapping experiments

To study the pattern of the elongation of the Cairns forked structures and rolling circles, pea ctDNA was mounted for electron microscopy under conditions of partial denaturation¹⁸. Ten partially denatured Cairns replicative intermediates were ordered with respect to their position in the denaturation map of pea ctDNA molecules that were an average of 22% denatured¹⁸. The positions of the replicated portions of these molecules with respect to the denaturation map are illustrated in Fig. 5a. These partially replicated molecules have a pattern that would be expected if they all had initiated replication at the same place and that both replicative forks were being extended at the same rate. The initiation site of these Cairns replicative

intermediates lies at the same point in the denaturation map where displacement loops (Fig. 1b) were shown to map⁷. The termination site for the Cairns round of replication appears to lie at a site 180° around the pea ctDNA molecule from the initiation site (Fig. 5a).

The pea ctDNA rolling circles have also been studied by denaturation mapping. In the partially denatured pea ctDNA preparations, rolling circles with two distinct patterns of denaturation on the tip of the tail were observed. An example of each type is presented in Fig. 3. The two types of rolling circles appeared in equal proportion and no other types of denatured tails were seen. The two partially denatured rolling circles of Fig. 3b and c are represented as a line graph in Fig. 3d. This representation shows that the tip of the tail of these two molecules is mapped at the same site and that each tail extends in the opposite direction from this site. The positions of 20 partially denatured tails with respect to the pea ctDNA denaturation map are illustrated in Fig. 5. All the tips of the tails are mapped at the same site and the tails extend in either direction on the map from this site. In the rolling circle model of DNA replication the tip of the tail marks the site where the initiation of replication takes place⁹. The initiation of rolling circle replication at a unique site in the pea ctDNA is similar to the results found for *E. coli* phage P2 replication except that the P2 rolling circles elongate in only one direction¹⁹. It should be noted that the pea ctDNA rolling circles all initiate replication at a site that is at or near the site where the Cairns round of pea ctDNA replication was found to terminate, which suggests a connection between the two processes.

Model for replication of chloroplast DNA

The results presented here suggest a model for the replication of pea and corn ctDNA. This model is illustrated in Fig. 1. The ctDNA replication is initiated by the formation of two displacement loops whose displacing strands are complementary to the opposite parental strands of ctDNA (Fig. 1b). The two displacement loops expand towards each other and initiate the formation of a Cairns replicative forked structure (Fig. 1c and d). This initial stage of ctDNA replication has been discussed in detail⁷. The small Cairns forked structures expand bidirectionally (Fig. 1e) until termination takes place at a site that is 180° around the circular ctDNA molecule from the initiation site. Separation of the daughter molecules takes place yielding two circular molecules that each have a single-strand break or small gap at the same site located in opposite daughter strands (Fig. 1f). This part of the replication process is not well understood in any system, but it probably yields open circular progeny which are then sealed into closed circles²⁰. In ctDNA the nicked circles could be sealed to closed circles or the 3'-OH of each nicked progeny molecule could be extended by a DNA polymerase molecule. This would displace a single-stranded tail (Fig. 1g) from the molecule and this tail could be filled in by discontinuous duplex synthesis to yield a molecule with a double-stranded tail (Fig. 1h). The tails might then be

converted to circular molecules by an intrastrand recombination event²¹. If both progeny from a Cairns round of replication initiated rolling circle synthesis, two types of rolling circles would be formed. In each case, the very tip of the tail would be mapped at the same site but the sequence of the two types of tails would extend in opposite directions from this site. This is the result that was observed in the denaturation mapping experiments with pea ctDNA rolling circles. Therefore, we feel that a Cairns round of replication is used to initiate a rolling circle round of replication in the ctDNA. A low frequency of Cairns structures was observed among the rolling circles that were shown to amplify the rDNA from *Xenopus laevis*²¹ and both Cairns structures and rolling circles have been observed during *E. coli* phage λ DNA replication^{22,23}, suggesting a similar model of replication for these two DNAs.

It is interesting that the ctDNAs from pea and corn, two evolutionarily divergent plants, have similar modes of replication. Why should the higher plant ctDNAs require two modes of DNA replication, one of which is an amplification mechanism²¹, and the other which is a duplication mechanism? Possibly it is related to a developmental aspect of higher plant chloroplast biogenesis that requires the rapid synthesis of a number of copies of ctDNA. The rolling circle mechanism might be used for this rapid synthesis of ctDNA while the Cairns mechanism might be used for normal duplication of ctDNA.

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letters to nature

Soft X rays from Sirius

ON April 3, 4 and 5, 1975 the star Sirius (α CMa) was observed with the soft X-ray detector aboard the Astronomical Netherlands Satellite (ANS). The instrument consists of a parabolic collecting mirror which has at its focus a proportional counter with a 3.6- μ m polypropylene window. It responds to X rays in the photon energy range 0.284-~0.2 keV. The projected area is 144 cm² which, allowing for reflectivity, counter efficiency

and so on, leads to a sensitive area of about 25 cm² at the carbon absorption edge (0.284 keV). The field of view is 34' FWHM circular. Further instrumental details are given in refs 1-3.

So that background could be subtracted, the observations were carried out in the offset pointing mode with the instrument alternately pointed at the source for 64 s and 40' away from the source for 64 s, the transition time being 16 s. Any ultraviolet contamination by light from the star can be determined by interposing into the light path a 0.5-mm MgF₂ filter which

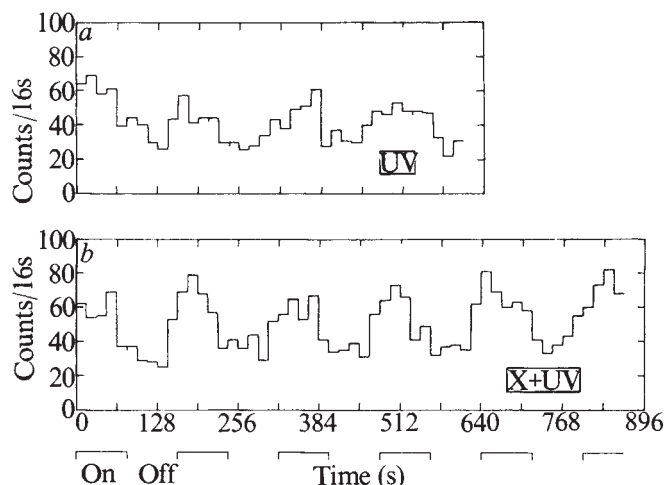
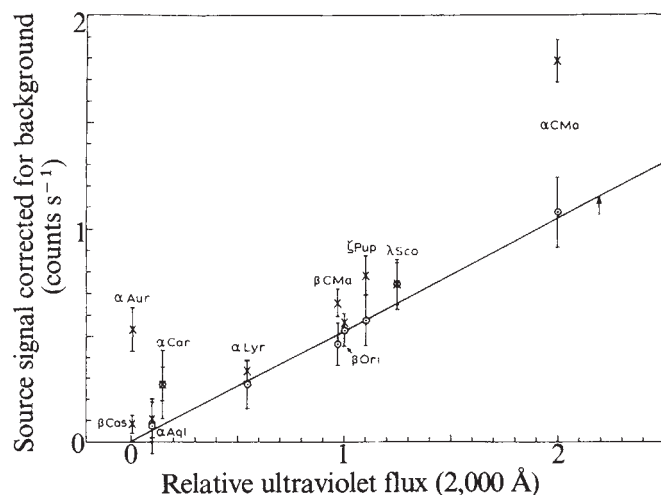


Fig. 1 Counting rates of Sirius measured on April 4, 1975 with ANS soft X-ray (0.25 keV) detector in offset pointing mode: a, 0920 UT; b, 0930 UT. UV (X+UV), signal measured with (without) MgF_2 filter; on (off), on (off) source.

blocks out the X rays but which transmits ultraviolet radiation above about 1,100 Å. The photons responsible for the ultraviolet contamination come from the wavelength region between about 1,600 and 2,500 Å (because of the transmission properties of the foil plus coating, ultraviolet radiation below 1,600 Å can be neglected). By measuring twice, with and without filter, X radiation (X) can be distinguished from ultraviolet radiation (UV). In that way the ultraviolet response was determined by observations on a number of bright ultraviolet stars (R.M. *et al.*, unpublished). Figure 1 shows typical measuring signals on Sirius, with and without the filter. In Fig. 2 the source signal, corrected for background and averaged over the total observation time of about 1,300 s, has been plotted against the relative ultraviolet flux. The total counting rate from Sirius, without the filter, that is, signal X+UV, is more than 6σ above the observed ultraviolet counting rate. The excess counting rate has been interpreted in terms of X-ray flux from that star system (R.M. *et al.*, unpublished). Figure 2 also shows that

Fig. 2 Counting rates of various ultraviolet stars as a function of the relative ultraviolet flux at 2,000 Å after subtraction of background. Vertical error bars are 2σ in length and represent the combined uncertainty in the source and background data. The excess signals $\{(X+UV)-UV\}$ of Sirius (α CMa) and Capella (α Aur) are 6σ and 5σ, respectively. ×, Signal without ultraviolet filter; ⊙, signal with ultraviolet filter; arrow, ultraviolet response.



Capella (α Aur), detected earlier as an X-ray star⁴, gives a signal 5σ above background.

The counting rates from the other stars depicted in Fig. 2 can be wholly attributed to ultraviolet radiation. The observed counting rate of 0.58 ± 0.10 counts s^{-1} and the instrumental sensitivity of 1.9 counts $(\text{photon cm}^{-2} \text{keV}^{-1})^{-1}$ give for Sirius an X-ray luminosity in the energy band 0.284–0.2 keV: $L_X = (9.1 \pm 1.6) \times 10^{27} \text{ erg s}^{-1}$. Sirius is a visual binary with at least two components: Sirius A (A1 V) and Sirius B (white dwarf). The rotation period is 50 yr, the present relative distance, a , of the two components is estimated to be about 30 AU.

The observations could be explained by invoking X-ray emission from a hot corona around Sirius A. Preliminary calculations show that such a corona would need to be heated up to a few million K. The energy required would exceed the convective energy by nearly two orders of magnitude, even considering the fact that the anomalous silicon abundance⁵ would yield an enhanced, soft X-ray line emission. It could be questioned whether the X-ray emission could be produced by accretion on to the white dwarf of a stellar wind from Sirius A. To generate an X-ray energy of, say, $10^{28} \text{ erg s}^{-1}$ (this a very conservative lower limit), the accretion rate $\dot{M}$ must be at least $10^{-15} M_\odot \text{ yr}^{-1}$ ($GM_B \dot{M} / R_B \approx 10^{28}$; $M_B = 1.05 M_\odot$; $R_B \approx 0.01 R_\odot$ (ref. 6), G is the gravitation constant). If it is assumed that the stellar wind speed is twice the escape velocity of Sirius A⁷, then (with $M_A = 2.2 M_\odot$, and $R_A = 1.75 R_\odot$; ref. 5, the accretion efficiency $(M_B/M_A)^2 (R_A/8a)^2 \approx 3 \times 10^{-10}$). Thus, a rate of mass loss from Sirius A of more than $3 \times 10^{-6} M_\odot \text{ yr}^{-1}$ would be needed to produce a total X-ray luminosity of $10^{28} \text{ erg s}^{-1}$. This is about 10^3 – 10^6 times the expected mass loss for an A-type main-sequence star⁸. Even the mass loss from the A2 Ia supergiant α Cyg, estimated from the Mg II resonance lines⁹, is only $\sim 3 \times 10^{-10} M_\odot \text{ yr}^{-1}$. Thus, the mechanism of accretion probably fails to explain the observed X radiation.

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Anomalous temporal behaviour of Her X-1

DURING January 1975 the Leicester Sky Survey experiment aboard Ariel V observed anomalous X-ray emission the during off state of Her X-1. Figure 1a shows the positive observations preceded by an extended period when no radiation was detected, a 3σ limit being shown by horizontal bars. The counting rates are averages over an entire satellite orbit and arise from photons in the range 2.4 to 19.8 keV. The counting rate scale may be converted to Uhuru units for a typical Her X-1 spectrum by multiplying by 1.27. Figure 1b shows that about half of the following on state was observed but not the actual turn on. If the turn on had occurred at phase 0.2 in the first observed cycle, the midpoint of the anomalous emission would have preceded the turn on by 16.5 d. A turn on extrapolated from

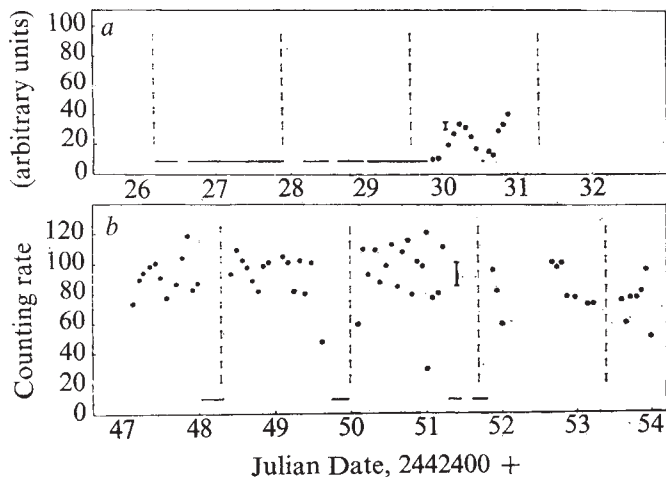


Fig. 1 Counting rate from Her X-1 during anomalous emission (a) and next regular on state (b). Vertical lines are mid-eclipse points, error bars are $\pm 1\sigma$ random errors.

Copernicus data (A. C. Fabian, private communication), taken two 35-d cycles later, reduces this to 14 d.

Although Her X-1 was extensively studied with Uhuru, observations were concentrated on the on state so that the probability of detecting emission during the off state was low. The only other indication of low state emission¹ from Her X-1 also occurred approximately half way through the 35-d cycle and was estimated to have a strength of between 0.3 and 1.0 times the typical strength during the on-state. Also, the emission we observed showed considerable variation in intensity and when our observations ceased was at 0.4 of the average on state strength and still increasing.

The mechanism of mass transfer from primary star to the compact companion in binary X-ray source is crucial in determining the emission behaviour of the source on long time scales. The identification^{2,3} of low mass star, HZ Herc, with the primary of the Her X-1 system suggests that the transfer is by Roche lobe overflow rather than by the stellar wind envisaged for the Krzeminski star/Cen X-3 system. In the former mechanism considerably more of the mass lost by the primary will be transferred to the secondary and the formation of the accretion disk is expected to be more orderly. Thus we anticipate that the long term temporal behaviour of Her X-1 will be simpler than that of, say, Cen X-3 where the density and ionisation state of the stellar wind have important roles in determining the activity and visibility of the X-ray emission⁴.

The regularity of the extended low states of Her X-1 point to a clock-like mechanism with which the marching of the pre-eclipse dip seems to be connected (D. Gerend *et al.*, unpublished). These dips may be due to obscuration of the X-ray source by material at the point where the overflow hits the accretion disk⁵ which itself may precess^{6,7} and periodically prevent us seeing the still emitting source. This explanation may be contrasted with that involving 'wobble' of the neutron star which either periodically alters the direction of a sharply emitted X-ray beam⁸ or actually causes the X-ray emission to be strongly modulated by giving rise to a geometrical mass gating action⁹.

The presence of radiation from Her X-1 within the extended low places an additional constraint on these models, especially if the radiation could be shown to be a regular feature of the off state and to be always at a particular phase of it.

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Whiskers and cosmic millimetre-wave sources

MANY extragalactic objects—N-type Galaxies, Seyferts and QSOs—are strong emitters of infrared and millimetre-wave radiation¹. For Seyfert galaxies the most likely model for the 2–20 μm radiation involves a central source of primary radiation surrounded by a shell of solid particles, the dust absorbing the primary radiation and re-emitting it in the infrared². There is independent evidence for the presence of dust in the nuclei of Seyferts including detections of differential reddening of [S II] lines³. The formation of dust could occur in the expansion phases following explosions of massive (or supermassive) stars⁴ and the primary radiation may be in the form of ultraviolet photons, soft X rays or low energy cosmic-ray nucleons⁵.

Dust particles involved for these models have so far been assumed to be similar to solid particles which are deduced to exist in our own Galaxy. Nearly spherical grains with radii in the range 10^{-6} – 10^{-5} cm could account for the observed 2–20 μm fluxes with reasonable mass requirements. A serious difficulty arises with regard to total grain mass if the same grains are to explain the observed millimetre and sub-millimetre wave radiation simply because of the low antenna efficiencies of very small particles at long wavelengths.

The total millimetre-wave fluxes of extragalactic sources and the peak wavelengths are somewhat uncertain. For Seyfert galaxies the average energy output at millimetre wavelengths is $\sim 3 \times 10^{44}$ erg s⁻¹ and for QSOs a range 10^{44} – 10^{46} erg s⁻¹ is likely, the spectra probably peaking at $\lambda \sim 120$ μm . This value of the peak wavelength indicates a minimum grain temperature $T_g \sim 30$ –50 K (depending on the wavelength dependence of the emissive efficiency). If T_g is the grain temperature at the outer edge of the shell, the flux in the range λ , $\lambda + d\lambda$ is

$$F_\lambda d\lambda \simeq \kappa_{\text{abs}}(\lambda) M_g \pi B_\lambda(T_g) d\lambda \quad (1)$$

where M_g is the total grain mass, $\kappa_{\text{abs}}(\lambda)$ is the mass absorption coefficient of grains, and $B_\lambda(T_g)$ is the Planck function. The maximum absorption efficiency of a small spherical particle being⁶

$$Q_{\text{abs}}^{\text{max}} \sim 10a/\lambda \quad (2)$$

in a typical case we find

$$\kappa_{\text{abs}}^{\text{(sph)}}(\lambda) \lesssim 30/4\lambda s \quad (3)$$

so that, taking $s = 2.2 \text{ g cm}^{-3}$,

$$F_\lambda d\lambda \lesssim 1.7 \times 10^{29} (T_g/\lambda^5) d\lambda (M_g/M_\odot) \text{ erg s}^{-1}$$

using equation (1) and the Rayleigh–Jeans approximation for $B_\lambda(T_g)$.

Taking $T_g \simeq 30$ K as a representative value, and setting $\lambda \simeq \Delta\lambda \simeq 1$ mm, we obtain

$$F_\lambda \Delta\lambda < 5 \times 10^{34} (M/M_\odot) \text{ erg s}^{-1} \quad (4)$$

for spherical particles. Thus, to explain a millimetre-wave output of 3×10^{41} erg s⁻¹ for a Seyfert nucleus we require grain masses $> 5 \times 10^6 M_\odot$; and for $F_\lambda \Delta\lambda \simeq 10^{44}$ – 10^{46} erg s⁻¹, in the case of QSOs, we require grain masses exceeding 2×10^9 – $2 \times 10^{11} M_\odot$. These grain masses must be regarded as untenably large.

These mass estimates are significantly reduced if long whisker grains of lengths 10^{-2} – 10^{-1} cm are present in the emitting dust shell. For cylinders with length $l \gg a$, and at wavelengths $\lambda \leq l$ the mean mass absorption cross section for randomly oriented particles is approximately⁷

$$C_{\text{abs}}(\lambda) = \frac{4\pi a^2 \sigma(\lambda)}{3c} \left\{ 1 + \frac{8K}{(K+1)^2 + 4(\sigma\lambda/c)^2} \right\} \quad (5)$$

where $\sigma(\lambda)$ is the optical conductivity, K is the dielectric constant, and a is the cross-sectional radius. The second term in curly brackets is in general negligible compared with the first, and for graphite, where optical data are available up to millimetric wavelengths⁸ $\sigma(1 \text{ mm}) \simeq 10^{15} \text{ s}^{-1}$. Thus equation (5) gives a mass absorption coefficient

$$\kappa_{\text{abs}}^{(\text{cyl})}(\lambda) \simeq 4\pi\sigma(\lambda)/3cs \quad (6)$$

where s is the specific gravity of grain material, and using equation (3) we have

$$\frac{\kappa_{\text{abs}}^{(\text{cyl})}(\lambda)}{\kappa_{\text{abs}}^{(\text{sph})}(\lambda)} > \frac{4\pi\sigma/3cs}{15/2\lambda s} \simeq 1.5(\sigma/10^{15}) (\lambda/10^{-4}) \quad (7)$$

For graphite with $\sigma(1 \text{ mm}) \simeq 10^{15} \text{ s}^{-1}$, $\lambda \sim 10^{-1} \text{ cm}$ we find the required mass estimates in the form of whisker grains are down by a factor $\sim 1.5 \times 10^3$. This gives grain mass estimates of $10^3 M_{\odot}$ for explaining the millimetre fluxes from Seyferts, and 10^6 – $10^8 M_{\odot}$ for QSOs. The implied hydrogen masses $10^5 M_{\odot}$ – $10^{10} M_{\odot}$ are now within the permissible range. Impure ice whiskers are expected to have a similar value of low frequency conductivity, so these mass estimates are also appropriate for ice whiskers, or formaldehyde polymer whiskers coated with impure ice.

The growth of formaldehyde whiskers in interstellar space has already been discussed by one of us^{9,10}. Crystal growth in whisker form is also well known to occur in the case of graphite condensing in suitable laboratory conditions^{11,12}. We will discuss the whisker growth process in greater detail elsewhere.

It may be argued that either formaldehyde polymers or graphite whisker growth could take place in conditions prevailing near the nuclei of Seyferts and QSOs. The former case arises where the ambient C/O ratio exceeds unity, and the latter where O/C exceeds unity. In the latter case, the oxygen uncondensed as H_2CO may be deposited as H_2O mantles on the formaldehyde whiskers. The maximum length l of whisker grains is determined by the condition for the exhaustion of condensable gas phase atoms in most cases of interest and is given by

$$l = (n_g/n_H)^{-1} (\pi a^2)^{-1} s^{-1} f m_A \quad (8)$$

Here f is the fraction of condensable atoms (by numbers) relative to H, m_A is their mass per molecule, a is the radius of the grains (or base sites on grains) which act as condensation nuclei, and s is the specific gravity of the condensing solid. For interstellar type grains with $a \simeq 10^{-8} \text{ cm}$, $n_g/n_H \sim 10^{-12}$, $s = 2.5$, $f \sim 10^{-3}$, $m_C = 12m_H$ we find $l \lesssim 1 \mu\text{m}$. Much longer particles could grow in the vicinity of galactic nuclei if lower n_g/n_H ratios and/or lower values of a are appropriate. Such situations could arise due to sputtering or evaporation of original grain nuclei in an explosive phenomenon, or simply from a low starting value of n_g/n_H . Grain lengths of $100 \mu\text{m}$ – 1 mm required for our radiation model may thus be justified fairly easily.

The whisker growth process is extremely rapid, and could easily occur within the time scales of protostellar collapse. The existence of whisker grains in galactic protostellar clouds would allow extremely bright far-infrared sources with very low dust masses. For example, the millimetre emission from OMC1

could be explained with whisker grain masses of less than $0.02 M_{\odot}$.

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Water in non-carbonaceous stony meteorites

WE report here on high-voltage electron microscope observations of the hydrous alteration products of olivine in an achondrite and an ordinary chondrite—two classes of meteorite in which hydrous minerals are rare. We argue that the Nakhla achondrite, and perhaps the Weston chondrite, contain water of extraterrestrial origin which was mobilised by mild shock deformation. The observations suggest that both meteorites have unusual volatile constituents.

Hydrous minerals are rare in meteorites other than types I and II carbonaceous chondrites¹. It has been pointed out that iddingsite occurs in the 'diopside'-olivine Nakhla achondrite (T. E. Bunch and A. M. Reid, 1974 Meeting of the Meteoritical Society). Iddingsite is a disequilibrium mixture of products of the hydrothermal alteration of olivine, in which approximate hexagonal close packing of the oxygen ions is retained². In the Nakhla achondrite it occurs mainly as red-brown veins in the large olivine grains. Using transmission electron microscopy we have examined a vein in material prepared by ion thinning³. The vein was found to have been formed as a result of alteration along a crack, which in turn is attributable to shock deformation since the adjacent olivine shows mosaicism and dislocation substructures characteristic of mild shock effects⁴. The fine-grained alteration product becomes colloidal towards the centre of the vein (Fig. 1a). The association with shock deformation supports the idea that the iddingsite is of extraterrestrial origin. The Nakhla achondrite is essentially an igneous rock which formed from a relatively oxidised melt, producing Fe^{+2} -rich silicates and primary magnetite; magmatic water is likely to have been present during its formation. The evidence for later mobilisation of the water by mild shock suggests that here leaching of rubidium may have been responsible for the radiometric anomalies^{5,6} in which whole-rock specimens deviate from the 1.24 Gyr mineral isochron believed to represent the crystallisation age⁵. The presence of water suggests that the parent body was not outgassed earlier than 1.24 Gyr BP.

The Weston chondrite has similar iddingsite veins in olivine. Where olivine with only slight mosaicism is crossed by narrow veins (less than $1 \mu\text{m}$ thick), electron diffraction patterns were obtained showing a topotactic transition to a goethite-like condition, similar to that described in terrestrial iddingsite². In the fine-grained matrix of the meteorite, we also found abundant iddingsite and similar material filling interstices. This accounts for the high content of bound water (0.98% by weight), in the bulk chemistry⁷. In Fig. 1b the relatively large crystal is olivine, with a rim of fine material which, by comparison with the vein occurrences, may be described as iddingsite. This material cements the grains in Fig. 1b. We found that the

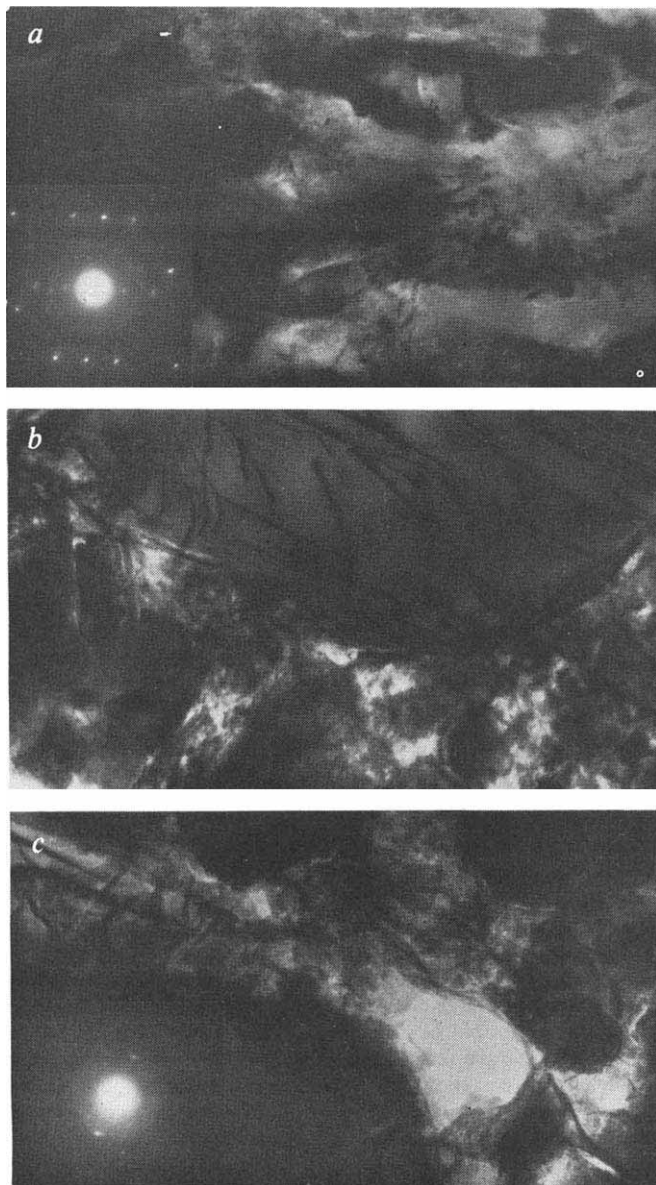


Fig. 1 High-voltage (1 MV) electron micrographs showing meteoritic iddingsite. Insets are diffraction patterns from the fine-grained areas. *a*, The Nakhla achondrite (BM 1913,26) $\times 18,000$ —diffraction pattern comprises spots from olivine and rings from the alteration product; *b* and *c*, the Weston chondrite (BM 1920,349)—*b*, $\times 13,000$; *c*, $\times 16,400$.

alteration makes an important contribution to the mechanical cohesion of the Weston chondrite. Where relatively coarse, the material often has a morphology comprising matted laths (Fig. 1c), and gave diffraction patterns dominated by rings corresponding to lattice spacings of 2.46 ± 0.04 and 1.49 ± 0.05 Å. Where strong, they were accompanied by other rings, consistent with patterns from randomly oriented crystallites of a clay phyllosilicate with $a \approx 5.2$ Å. Smaller grains tended to have equidimensional images, and diffraction gave pseudo-hexagonal arrays of diffuse spots comparable with basal diffraction patterns of the disordered phyllosilicate constituent in terrestrial iddingsite².

The Weston chondrite is mildly shock-lithified (J. R. A. and D. J. Barber, unpublished). If the water was present during the shock events, it would have been mobilised and could thus have produced the observed alteration. The possibility that the hydration occurred later should, however, be considered. The Weston chondrite (like the Nakhla achondrite) is an observed fall and, therefore, has not suffered normal terrestrial weathering, but it is reduced and porous, like the Apollo 16 'rusty rocks' in which hydrous minerals are thought to have

been produced through the hydrolysis of primary lawrencite, FeCl_2 , by terrestrial atmospheric water vapour⁸. The Weston chondrite contains, however, an order of magnitude more water than do the Apollo 16 rocks, and to attribute this to the lawrencite reaction requires an exceptionally high initial content of the volatile element chlorine; abnormal enrichment of extraterrestrial volatiles is found in the Apollo 16 rocks themselves⁸.

Weston is a 'gas-rich' chondrite⁹, which means that some individual grains suffered unshielded irradiation by the solar wind, resulting in the implantation mainly of hydrogen and helium. The observed alteration cannot, however, be attributed to the formation of water by reaction between mineral grains and solar wind ions, as lunar surface breccias are generally anhydrous. If aggregation of the grains occurred on a small atmosphere-free body^{10,11}, a magmatic source of reactive volatile elements is also unlikely. On the other hand, a likely source is a 'dust' component of type I and/or type II carbonaceous chondrite composition. Macroscopic inclusions of carbonaceous chondrite are certainly present in some gas-rich meteorites¹⁰. These meteorites probably formed during the terminal stages of accretion of their parent bodies, in an environment that was favourable for the incorporation of such inclusions^{10,11}. Moreover, a carbonaceous chondrite component would carry water, because it would consist largely of hydrous phyllosilicates¹. Because the hydrous minerals in the Weston chondrite were found on cracks and grain boundaries, it is unlikely that they could be unaltered grains from a carbonaceous chondrite source though such a source could have supplied the water for the subsequent alteration. If all the water in the Weston chondrite is from this source, the indicated minimum content of type I carbonaceous chondrite is 5% by weight, using the figure of 20% for the water content of type I material¹; for type II, the corresponding minimum content in Weston is 7.5% by weight. These are minimum figures because water may have been lost during accretion. If the Weston chondrite does contain more than 5% of a carbonaceous chondrite component, it should be identifiable chemically. Neither the major element data⁷ nor the Kr and Xe isotopic abundances¹² are, however, diagnostic of its presence. An attempt is being made to characterise any unusual component which may be present in the Weston chondrite. Until this has been done, the additional possibility that the Weston chondrite is from a body which had a hydrous atmosphere must also be left open. Whatever the source of the water, the pervasive alteration suggests that the matrix of the Weston chondrite incorporated an unusually large amount of chemically reactive material.

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Lag times for oceanic responses to climatic change

RECENT studies of deep-sea sediment cores have defined changes in surface water circulation¹, in continental ice volume as recorded by oxygen isotope variations in foraminiferal tests², and in the corrosiveness of near-bottom water to calcium carbonate^{3,4} during the past million years. These changes are all attributed to the major climatic changes that have occurred during the Quaternary^{1,2,5,6}.

Few of the studies have attempted to establish whether the various changes are synchronous, or whether there are leads and lags in the system⁷. Such information is critical in evaluating theories and models of climatic change, and in sorting out causes from effects. Thus, if bottom water changes precede surface water and ice volume changes during deglaciation, models like that of Newell⁸ are favoured, whereas if the ice volume decreased first, an albedo-related cause is more likely.

To determine sequences of change, either extremely accurate time scales for the records of the oceanic system and the continental ice volume, or records of the two systems from the same core samples are needed. The latter approach eliminates problems of correlation or inaccuracies of time scales in different deep-sea cores, and is the approach used here.

The sediment core selected for detailed study (core Y69-106P, 2°59'N, 86°33'W, 2,870 m) was taken in the eastern equatorial Pacific, an area where the surface circulation varied significantly from glacial to interglacial times⁹. To establish a time scale throughout the core, a sedimentation rate model was constructed assuming that the rate of accumulation of quartz has been constant at the core location (a reasonable assumption given the depositional regime in the area¹⁰). The fact that calculated accumulation rates of quartz were constant to within 3% between points dated by three carbon-14 determinations, an ⁴⁰Ar/³⁹Ar age, and oxygen isotope and biostratigraphic boundaries of known ages, supports this assumption. Details of the model are given in ref. 10. Measurements of carbonate, opal, and quartz concentrations together with bulk density measurements were transformed by the model to give absolute accumulation rates (g cm⁻² 10³ yr) for the total sediment, calcium carbonate, opaline silica, and the remaining (non-biogenic, non-quartz) detritus during the past 450,000 yr.

Conversion of the raw calcium carbonate analyses to accumulation rates eliminates the problems inherent in working with concentration data. In the Pacific, changes in the concentration of calcium carbonate probably reflect changes in accumulation rate of carbonate particles, but in the Atlantic, fluctuations in

Fig. 1 Calcium carbonate and opaline silica accumulation rates (in g cm⁻² 10³ yr) and oxygen isotopic data from core Y69-106P.

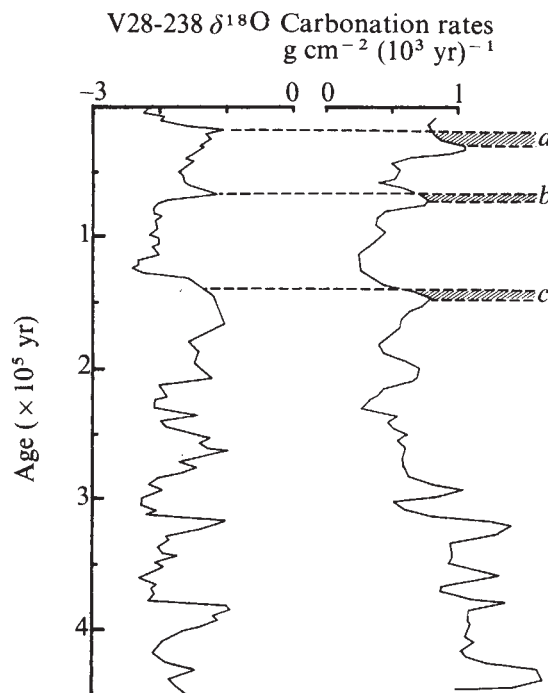
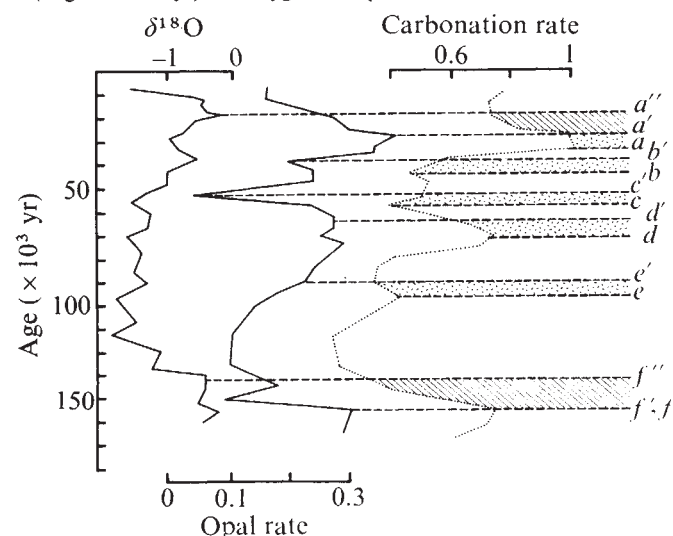


Fig. 2 Calcium carbonate accumulation rate in core Y69-106P compared to oxygen isotopic analyses from core V28-238.

carbonate concentration are strongly influenced by variable dilution as a result of changes in the accumulation rate of terrigenous material¹¹.

Comparison of the silica and carbonate accumulation rate curves and the oxygen isotopic composition of foraminiferal calcite in Y69-106P (Fig. 1) suggests that climatic and oceanographic changes have not been synchronous. The accumulation rate curves for opaline silica and calcium carbonate during the past 150,000 yr are very similar (Fig. 1), but there seems to be a slight lag between the two curves during this period, changes in calcium carbonate accumulation preceding changes in opal accumulation rates. The calcium carbonate and opaline silica accumulation rates seem to be responding to the same changes in the driving mechanism, presumably climate, but the response times are different. The average lag time between change in carbonate and opal accumulation rate (Fig. 1a-f) is 2,600 yr.

The relation between the isotopic data and the other parameters is less apparent, but again the carbonate rate curve appears to lead, this time by 5,000–10,000 yr. Confirmation of this lead is provided by a comparison of the carbonate curve of Y69-106P with a more complete isotopic record from western Pacific core² V28-238 (Fig. 2). For the last three glacial maxima, the carbonate peak precedes the isotopic (ice volume) peak by about 5,600 yr.

Opal accumulation rates seem to be controlled by biological productivity in the surface waters^{10,12} whereas changes in the carbonate accumulation rates reflect changes in the dissolution of calcium carbonate by bottom waters³. Thus the lags between changes in the calcium carbonate accumulation rates, the opaline silica accumulation rates, and the oxygen isotope analyses from core Y69-106P indicate that the following sequence of events occurred in the eastern equatorial Pacific during the transition from the most recent glacial to the present interglacial period (Fig. 1, a, a', a''):

An increase in corrosiveness of the bottom water (indicated by a reduction in calcium carbonate accumulation rates), preceded a decrease in surface productivity (indicated by a reduction in opal accumulation rates), which in turn preceded the recession of continental glaciers (indicated by a reduction in the ¹⁸O content of foraminiferal carbonate). The period covered by this sequence of events was about 5,600 yr with

about 2,600 yr (an average of events *a-f* Fig. 1) elapsing between the bottom water and surface circulation changes and 3,000 yr between the change in surface circulation and the recession of the continental glaciers. Because of the relatively wide spacing in time of individual samples from Y69-106P (the average sample interval is about 4,000 yr) these estimates represent maximum lag times.

If the lags between the carbonate and opal accumulation rate curves were random and independent, the probability of the observed succession in the six events *a-f* in Fig. 1 would be 0.016—highly unlikely. Rather, the observed changes seem to be systematic and are consistent with two recent models^{8,13} of climatic change. Newell⁸ has suggested that the termination of a glacial period is first marked by a decrease in the rate of formation or even stagnation of bottom waters (this would result in an increase in dissolved carbon dioxide in the bottom waters, leading to accelerated carbonate dissolution). He further suggests that the bottom water stagnation would be succeeded by reduced upwelling (leading to reduced opal deposition) and finally by wasting of continental glaciers. The data from core Y69-106P are in complete accord with Newell's model. If confirmed by data from other geographical areas, these results support continued development of the model and seem capable of providing accurate estimates of the lag times involved in the change of the oceanic and cryospheric systems from glacial to interglacial modes.

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Strain resulting from adhesive action of water in capillary bridges

WE report here on the measurement of adhesive forces of thin films of water between glass plates. Our approach differed

Fig. 1 Displacement, db , in a deformable isolated capillary. (From surface-energy considerations.)

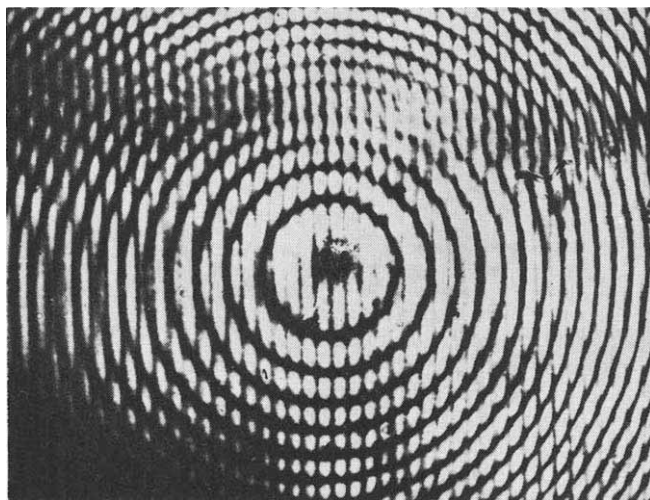
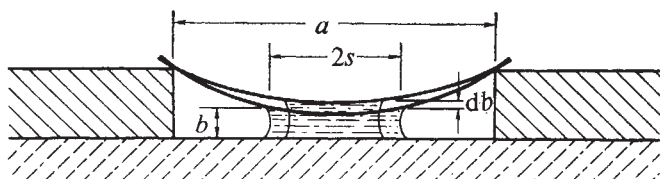


Fig. 2 Interference fringe pattern obtained by a mechanical loading of 20,482 dyne.

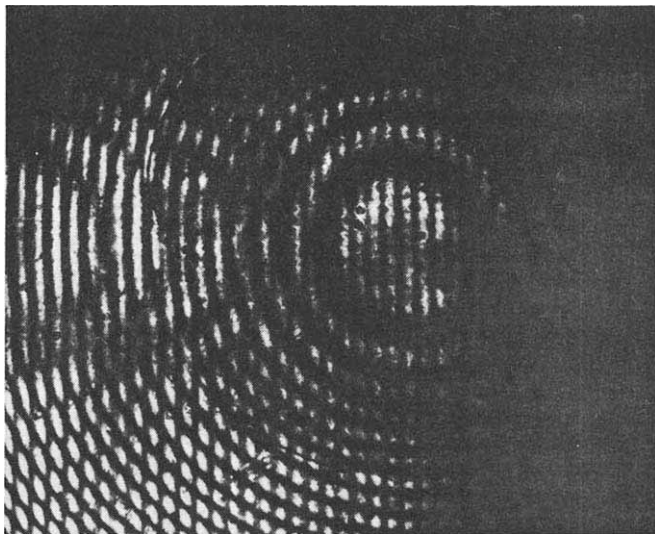
from the traditional method of measuring the force necessary to rupture the liquid film in tension¹⁻³; we used interferometry to measure the elastic strains produced around the bridges. The main purpose of the study was to assess the degree of agreement between measured adhesive forces and those predicted by classical capillary theory.

When a surface-energy consideration is applied to a liquid bridge between a deformable capillary wall and a rigid capillary wall, the work done by the force F during an increase, db , in the distance b (Fig. 1) is equal to the energy of wetting and the elastic bending energy of the capillary wall:

$$Fdb = (\gamma_{SV} - \gamma_{SL})dA_{SL} + \gamma_{LV} dA_{LV} + dU \quad (1)$$

where γ is an interfacial energy, A an interfacial area, and the subscripts S, V and L denote solid, vapour and liquid, respec-

Fig. 3 Interference fringe pattern obtained by capillary adhesion. An adhesive force of 9,510 dyne is measured.



tively; U is the bending energy in the capillary wall. From the Young-Dupre equation, $\gamma_{LV} \cos \theta$ can be substituted for $(\gamma_{SV} - \gamma_{SL})$ with θ being the contact angle of the liquid on the

capillary wall. The theory of bending of plates⁴ gives:

$$dU = Fdb/\cos(\pi/a) \quad (2)$$

When $s \gg b$, the term $\gamma_{LV}dA_{LV}$ is negligible, and by using β to represent π/a equations (1) and (2) can be combined to give

$$\frac{F}{\pi s^2} \left(1 + \frac{1}{\cos \beta}\right) = \gamma \cos \theta \frac{2}{b}, \quad (3)$$

which indicates a linear relationship with a slope of $\gamma \cos \theta$.

A helium-neon laser beam was directed normal to two parallel glass capillary walls separated by a metal spacer 2 μm thick. Deflections of the deformable wall under applied forces gave rise to interference fringe patterns which are linearly related to the magnitudes of the applied forces. Mechanical loading was used to produce fringe patterns for calibration (Fig. 2).

Fringe patterns were obtained with water bridges having diameters of 0.04, 0.070, 0.12 and 0.13 cm (Fig. 3). Next, the forces of adhesion were calculated based on the calibration.

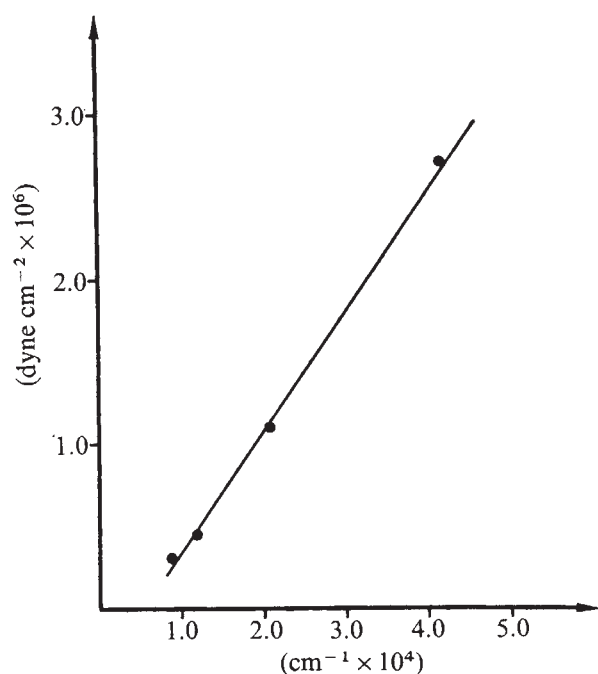


Fig. 4 The relationship of $(F/\pi s^2)(1+1/\cos \beta)$ (ordinate) and $2/b$ (abscissa) for a deformable glass-water capillary system. The experimental value of $\gamma \cos \theta$ is 73.9 dyne cm^{-1} , compared with a predicted value of 72 dyne cm^{-1} .

Using these results, Fig. 4 was plotted. The slope of the linear regression line is 73.9 dyne cm^{-1} which, from equation (3), would be equal to γ since θ was near zero. This close agreement with the surface tension value of water supports the correctness of equation (3). The results indicate that the surface energy of wetting can account for the elastic strains observed.

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Three-coordinated magnesium in dehydrated offretite

OFFRETITE is a rare natural zeolite whose synthetic equivalent, zeolite O, is commercially important as a molecular sieve¹. Its aluminosilicate framework is topologically related to that of erionite², an abundant natural zeolite³. The catalytic properties of molecular sieve zeolites have been ascribed to framework-bound protons, strong electrostatic fields, and oxygen vacancies in the framework⁴. As befits the small radius of Mg^{2+} ions (0.049 nm for four-coordination, 0.072 for six-coordination and 0.089 for eight-coordination)⁵, Mg normally occurs in six-coordination with oxygen and hydroxyl, though four, five and eight-coordinations occur infrequently (for example, four-akermanite: $\text{Ca}_2\text{MgSi}_2\text{O}_7$, $\text{Mg}-\text{O} = 0.1876$ nm; five-grandidierite: $(\text{Mg},\text{Fe})\text{Al}_3\text{O}_4\cdot\text{BO}_4\cdot\text{SO}_4$, 0.2176, 0.2054, 0.2040, 2×0.1970 ; farringtonite: Mg_3PO_4 , 0.196, 0.197, 0.201, 0.206, 0.214; eight-pyropo: $\text{Mg}_3\text{Al}_2\text{Si}_3\text{O}_{12}$, 4×0.2196 , 4×0.2342)⁶. We report here on the occurrence of three-coordinated Mg in dehydrated offretite, which should produce a strong electrostatic effect on adjacent sorbed molecules.

Table 1 Atomic coordinates

	x/a	y/b	z/c
Si(1)*	0.9998(3)	0.2277(2)	0.2177(2)
Si(2)	0.0968(3)	0.4273(3)	0.5
O(1)	0.0408(6)	0.3511(5)	0.3182(9)
O(2)	0.0896(3)	0.9104(3)	0.2783(10)
O(3)	0.8722(9)	0.1278(4)	0.2879(11)
O(4)	0.0030(9)	0.2483(7)	0
O(5)	0.2425(6)	0.7575(6)	0.5
O(6)	0.4592(7)	0.5408(7)	0.5
Ca	0	0	0.5
K	0.4964(12)	0.5036(12)	0
Mg	0.333	0.667	0.5

*Numbers in brackets are atom designation numbers.

Hydrated offretite⁷ ($\text{K}_{1.1}\text{Ca}_{1.1}\text{Mg}_{0.7}\text{Si}_{12.8}\text{Al}_{5.2}\text{O}_{36} \cdot 15\text{H}_2\text{O}$) contains a K ion in each cancrinite cage, a pentahydrate of Mg in each gmelinite cage, and hydrated Ca ions in the main channels. Electron microprobe analysis of a new crystal, from Mount Simiouse, France, showed that this had $\text{K}_{1.04}\text{Ca}_{1.04}\text{Mg}_{0.95}\text{Al}_{5.2}\text{Si}_{12.8}\text{O}_{36}$ atoms in each cell. The crystal was dehydrated in a silica capillary for 22 h at 500 °C and 10^{-5} torr before sealing. Using $\text{MoK}\alpha$ radiation, 4,700 intensity measurements at room temperature yielded 514 independent non-zero diffractions in $\text{P6}_3\text{m2}$, a 1.3229(5), c 0.7338(4) nm. Least-squares refinement yielded the coordinates shown in Table 1.

The stereo plot (Fig. 1) shows Mg occupying the centre of a single six-ring where it is bonded to three oxygens at 0.208(1) nm. The six-ring is very distorted to accommodate this short distance and the other three oxygens are displaced outwards to 0.288(2) nm. Some weak bonding may occur to these three oxygens, but the strong disparity in the distances implies that the Mg is effectively three-coordinated. Each Ca ion lies between two six-rings which are distorted so that three oxygens from each give near-octahedral coordination at 0.2619(8) nm. Each K ion lies at the middle of a distorted eight-ring with four oxygens at 0.3258(7) as nearest neighbours and two more at 0.3350(6) nm. All the cation-oxygen distances are longer than the values expected from simple ionic theory and distances found in most silicates (for instance, $\text{Ca}-\text{O} = 0.24$; $\text{K}-\text{O} = 0.27$; and predicted $\text{Mg}-\text{O}$ for three-coordination is less than 0.19 nm). The mean Al, Si-O distance of 0.1631 nm is less than the value of 0.165 obtained by interpolating the Al/Si ratio of offretite between the data for anhydrous framework silicates⁸. This suggests that the bonding in the framework of the dehydrated zeolite is stronger than in normal silicates and that the bonding

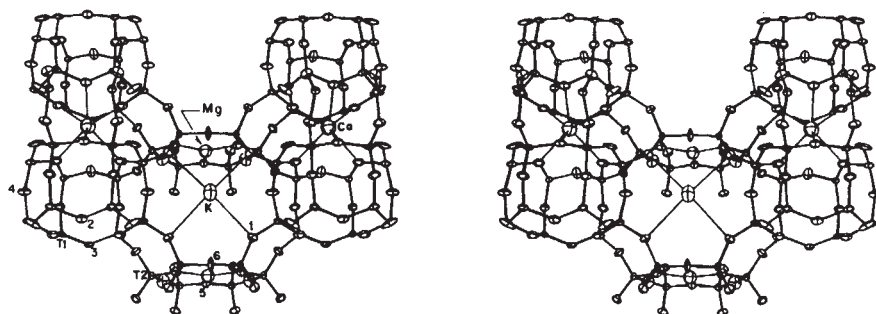


Fig. 1 Stereoplot of dehydrated offretite. Ellipses drawn at 30% probability level.

between extraframework cations and framework oxygens is weaker.

The Mg ion of dehydrated offretite should have a strong electrostatic field around it, and sorbed molecules should be strongly attracted to form a complex. Perhaps catalytic properties would result. In the dehydrated structure⁹ of mazzite, which is another zeolite, Mg was tetrahedrally coordinated to three oxygens of a six-ring plus a fourth oxygen species which might be either a water molecule or a hydroxyl ion. Perhaps addition of one water molecule to each Mg ion would result in a similar coordination in offretite. Disproportionation of a water molecule into a hydroxyl ion and a proton could occur, with the latter condensing with a framework oxygen to yield a framework hydroxyl. The latter might be a Brønsted-acid catalyst (see, for example, ref. 4).

The unusual coordination of Mg in dehydrated offretite results from the spatial restrictions posed by the topology and geometry of the aluminosilicate framework. The four-rings cannot accommodate the Mg ion because of electrostatic repulsion from the Al, Si ions, and the centre of a six-ring offers the best of a set of bad options. Because of spatial restrictions the six-ring cannot pucker sufficiently to yield a near octahedral coordination, though it can pucker to give a triangular coordination. Thus the Mg ion, which would have had a reasonable coordination in the hydrated zeolite, becomes forced into an unusual coordination. A similar situation occurs in dehydrated Type A zeolites, in which transition metal ions have been found in three-coordination at or near the centre of six-rings^{10,11}.

Full details, including the nature of an erionite intergrowth, will be published elsewhere.

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Effect of glass dissolution on corrosion measurements

BOROSILICATE glass is widely used in electrochemical and corrosion studies because of its transparency, rigidity, ease of fabrication and, in particular, its resistance to chemical attack. Some disadvantages of the use of glass at very high temperatures, such as its solubility and the risk of solution contamination in autoclave studies of aqueous electrochemical processes, have been pointed out by Jones and Masterson¹. In a previous communication² from this laboratory it was reported that at temperatures of 80 °C and above, appreciable dissolution of silica from glass can occur and that, in the corrosion of zinc, appreciable amounts of zinc pyrosilicate hydrate are present in the corrosion products. Later³, figures for the silica contamination of distilled water held in glass vessels at 90 °C were quoted and the possible implication of glass dissolution on the corrosion behaviour of mild steel discussed.

Contamination of aqueous solutions by the test vessel is obviously undesirable in the acquiring of any standard corrosion data and this is particularly so in corrosion rate studies, since silicates—especially metasilicates—have an appreciable inhibitive action on metallic corrosion even when present in low concentrations. Since most of the experiments on the influence of temperature on corrosion rate—at least up to 100 °C and on occasions even above—have been conducted using glass test vessels considerable doubt must be placed on the validity of data obtained in this way.

Comparisons have been made of the corrosion behaviour of abraded mild steel sheet in distilled water at 90 °C using in the one case new 200 × 35 mm test tubes and condensers in borosilicate glass, as normally supplied commercially to laboratories, and in the other case equipment of similar geometry made in transparent fused quartz. Test specimens 30 × 25 × 0.8 mm were fully immersed in 60 cm³ of triply distilled water (pH 5.5, conductivity 84 μS m⁻¹) with the specimens resting on the bottom of the tubes at an angle of about 30° to the vertical. A slow stream of washed air was bubbled through the water just below its surface throughout the test. Specimens were tested in duplicate in separate vessels for periods of 28 and 100 d with the temperature maintained at 90 ± 1 °C throughout the test period.

Within the first few hours of immersion the specimens in glass were subjected to highly localised attack with hardly any rust in the water, whereas those in the quartz tubes had undergone more general corrosion, with the production of an appreciable amount of suspended rust. This difference in appearance of the specimens and the waters in the two types of vessel was maintained throughout the 100-d test period (Fig. 1). Measurements of dissolved monomeric silica, as determined by the colorimetric molybdate method, made on water after 8 d in similar tubes but without specimens present gave values of ~10 p.p.m. SiO₂ in water from glass but <2 p.p.m. SiO₂ in

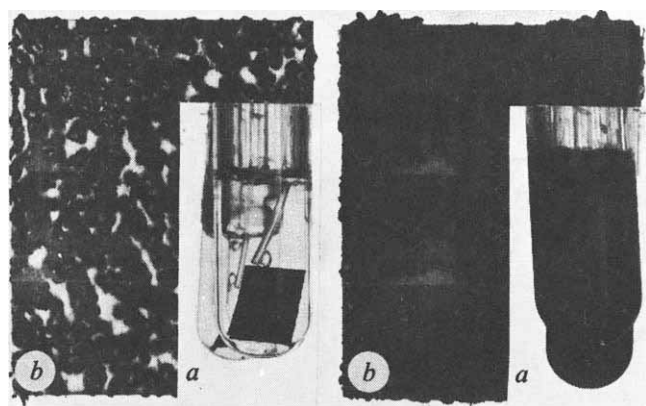


Fig. 1 *a*, Mild steel specimens in quartz and glass tubes after 28 d at 90 °C. Right, quartz tubes and "rusty" water; left, glass tubes and clear water. *b*, Specimens after removal from these tubes.

water from quartz vessels. Corrosion product was removed from specimens after test by cathodic treatment in 5% H_2SO_4 + 0.1% quinoline at 75 °C. Curves of weight loss as a function of time are shown in Fig. 2 and the appearance of typical specimens after removal from the test vessels but before removal of corrosion product are shown in Fig. 1.

We suggest that the presence of enhanced quantities of silica in the water in new glass vessels has resulted in a partial inhibitive effect on the corrosion process. The reason for the higher rate of silica dissolution from glass than from pure silica (quartz) is presumed to be the result of an initial dissolution of alkali metal ions from the glass producing local alkalinity which will facilitate subsequent dissolution of the Si-O network. The corrosion effects observed are, however, not thought to be associated with pH changes in the bulk of the water since the maximum pH values found in glass vessels—without specimens present—were only 7.1 after 28 d and 7.6 after 100 d.

These results have wide implications for corrosion tests in hot waters and will influence conclusions relating to the effect of temperature on the corrosion behaviour of steel, and pro-

bably other metals, in these conditions. Because of the attenuation of corrosion rate with time shown in these experiments, the effect of glass dissolution may have a considerable bearing on the value, or even the existence, of a temperature for maximum corrosion below 100 °C.

Following the 28-d tests the glassware was cleaned in hot HCl, followed by thorough washing in water, and the tests repeated. The corrosion rates then measured over 28-d were similar to those obtained in quartz vessels, and furthermore, the amounts of silica dissolved from these used vessels were lower than those from new vessels. In view of the uncertainty of the effectiveness of any conditioning treatment, however, we consider it inadvisable to rely on this approach to avoid silica contamination of hot waters. We therefore recommend the avoidance of glass vessels for any accurate determinations of corrosion rates in such conditions.

Further work is in hand to determine the magnitude of the effects observed at temperatures below 90 °C.

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Unique form of tool-using in two gastropod molluscs (Trochidae)

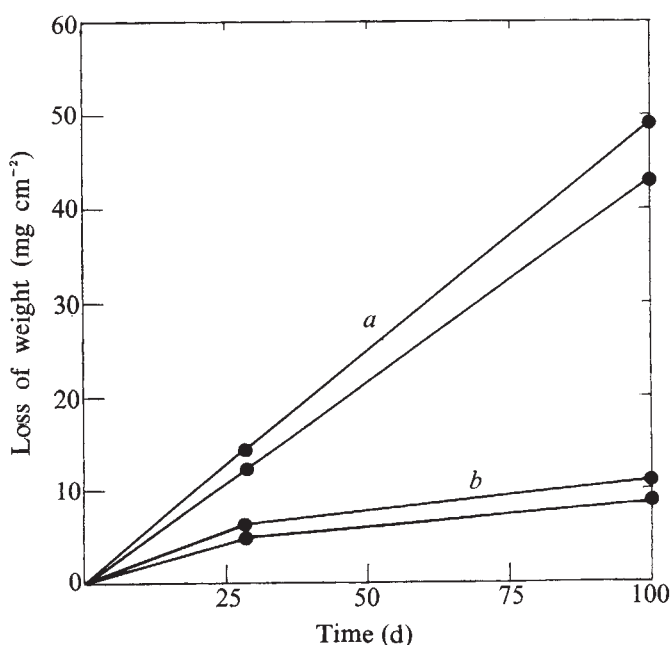
TOOL-USING, the ability to manipulate an object as a functional extension of the body, has been described in a number of vertebrate and invertebrate species¹. Although earlier workers regarded it as an indication of insight learning, its significance as a behavioural adaptation^{2,3}, possibly arising through the novel use of a pre-existing behaviour pattern⁴, has been stressed in recent studies.

We report here a form of tool-using in two species of prosobranch gastropod, *Tegula brunnea* and *T. funebralis*, both of which inhabit the intertidal region on the Pacific coast of the US. This behaviour, unlike previously reported instances of tool-using, functions to alter the position of the organism itself rather than displace or modify an extraneous object, and to the best of our knowledge has never been observed in nature or reported from other laboratory studies.

Approximately 30 specimens of both species of *Tegula* (5.8-11.9 g) were collected by the Peninsula Marine Biological Company, Sand City, California, and maintained in our laboratory in a 30 gallon refrigerated Instant Ocean aquarium at 10 °C. They readily fed on brown kelp (*Egregia*).

When the snails were inverted, the propodium was extended over the edge of the shell and engaged in a brief period of probing. If they had access to a solid substrate or an object heavy enough to support their weight, the propodium adhered to the support and pulled the shell over in a manner similar to that reported for other gastropods^{5,6}. A gravel substrate consisting of limestone pebbles having an average weight of 0.23 g and an average diameter of 6.0 mm could not sufficiently support the weight of an inverted snail. The prehensile anterior tip of the propodium would grasp a stone and lift it from the aquarium floor. Instead of releasing it immediately, however, the stone was conveyed in a posterior direction to the end of the foot through a series of alternate, retrograde undulations of the sole. This pattern is also typical of the normal locomotory movements of these species⁷. More stones were similarly procured and transferred to the posterior and median portion of the foot where they were accumulated (Fig. 1*a*). The continued

Fig. 2 Corrosion-time curves for abraded mild steel in distilled water at 90 °C. *a*, Specimens in quartz tubes; *b*, specimens in glass tubes.



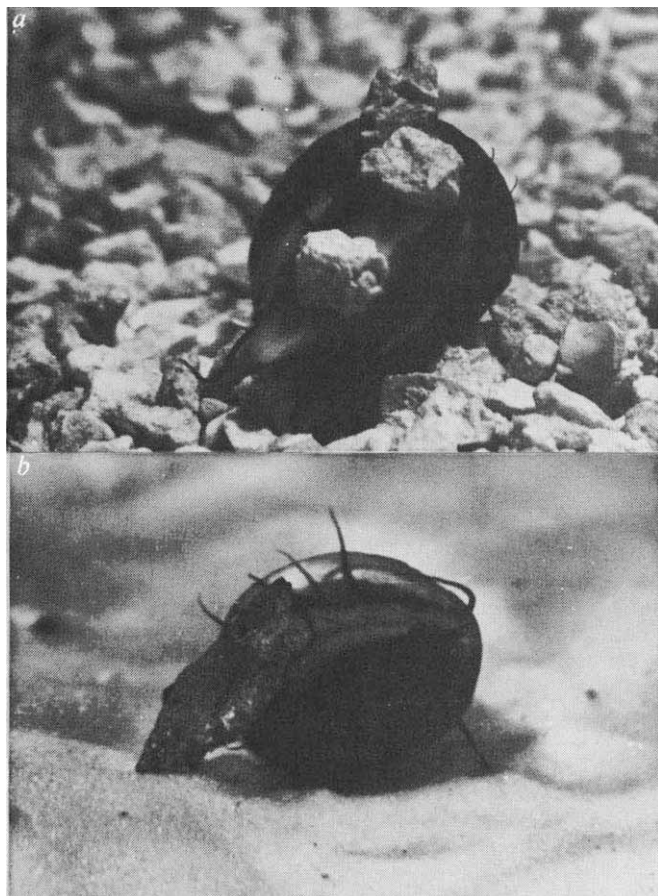


Fig. 1 *a*, Inverted specimen of *T. brunnea* in the process of transferring small stones to the posterior portion of the foot where they are accumulating. Note that the propodium is flexed downward and is ready to probe the substrate for another stone. The diameter of the widest whorl is approximately 2.5 cm. *b*, Inverted specimen of *T. brunnea* that is using a small flat stone as a shovel to gain the leverage to right itself on the sandy substrate.

upward flexion movements of the propodium in acquiring stones and the rotating movements of the snail body with the additional weight afforded by the retained stones resulted in the gradual displacement and eventual righting of the snails. If larger stones approximately 35% of the snail's weight were selected by the foot and extended to the side, the shell was induced to roll and the snail was promptly overturned. When an elongate stone was used, righting occurred by pushing it against the substrate in a shovelling fashion during the contortions and twisting movements of the snail body (Fig. 1*b*). Thus, these species of *Tegula* achieved the leverage necessary to bring the foot closer to the substrate by shifting their centre of gravity upward by the added weight of the retained stones and were able to restore themselves to an upright position.

The righting efficiency was affected and perhaps influenced by the nature of the supporting substrate, the position of the shell on the substrate, and the size of the stones used. There was little or no difference between the overall behavioural pattern of the two species, observed on approximately 60 occasions.

Both *T. brunnea* and *T. funebris* possess a relatively heavy, turban-shaped shell which tends to overturn them after they have been dislodged from the substrate. The righting ability of these species of *Tegula* is significant as inverted snails unable to right themselves could not feed and would be particularly subject to predation by carnivorous echinoderms such as *Pisaster*, to which these species of *Tegula* respond by rapid flight movements⁸.

T. funebris, which has a patchy distribution on the Pacific coast of the US, is found in large numbers on moderately exposed

rocky shorelines and is only encountered rarely in sandy areas⁹. At Mukkaw Bay, Washington, *T. funebris* is particularly abundant in areas in which small cobbles and pebbles are present rather than solid bench rock^{10,11}. From our observations of the Washington state coastline a few years ago, these small cobbles are several centimetres larger than the small stones used in our aquarium study. But as the snails move down the beach they may encounter substrata on which stone manipulation may have some adaptive significance. This would be particularly true in areas in which small stones or gravel would accumulate—for instance, on the bottoms of tidal pools and between larger rocks.

It has been suggested that the large operculum of strombid gastropods may have evolved partly to compensate for the lack of a large, long foot which could be used for righting movements⁷. We suggest that the stone manipulation as a form of tool-using may have similarly evolved as a compensatory behavioural adaptation.

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Significance of dorsoventral abdominal vibration among honey-bees (*Apis mellifera* L.)

IN observation hives worker honey-bees may often be seen to perform a behaviour pattern called dorsoventral abdominal vibration (DVAV)¹, the jerking dance², or shaking³. It has been suggested^{4–6} that it serves to prepare bees for flight, mainly on the grounds that the only occasions on which queen honey-bees are vibrated (subjected to DVAV) are before their orientation and mating flights and before swarming. But conclusive evidence has not been provided, and von Frisch regards the function of DVAV as still being obscure³. Gahl⁷ also leaves this question open.

The African bee, *Apis mellifera adansonii* L., is eminently suitable for study because, unlike European races, it has the habit of absconding if the nesting site becomes unfavourable for some reason. If DVAV does promote flight it should reach its highest level in a colony about to abscond, as this represents an extreme case of flight activity in which all the bees leave the nest together.

Detailed observations were made on two colonies in two-frame observation hives, one of which consisted of about 4,500 workers and the other of about 3,000. On the day of absconding the queens were not vibrated at all and the frequency of DVAV among the workers was extremely low. During numerous scans of the whole hive usually only one bee, and often none, could be seen performing DVAV at any time. DVAV is thus not a prerequisite for flight of either queens or workers.

This conclusion received support from observations made possible by another behavioural characteristic of the *adansonii* bee. In favourable temperature conditions foragers may be active at very low light intensities and the number leaving a hive often reaches the diurnal peak shortly after first light. In a colony of approximately 3,500 bees this exodus of foragers was compared on two occasions with the first DVAV performances

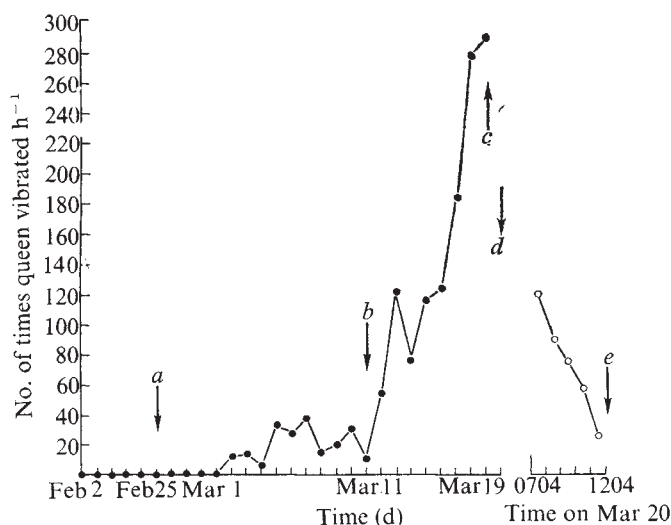


Fig. 1 Frequency with which a queen honey-bee was vibrated during a swarming cycle. *a*, First queen cup cell constructed. *b*, First egg laid in a cup cell. *c*, First queen cell sealed. *d*, Colony swarmed on March 20, 1975. *e*, Queen departed with swarm at 1204. The observation hive contained three Langstroth brood frames and the bees numbered 9,500–10,000 at the time of swarming. DVAV frequency was sampled for 90 min each day until the first queen cell was sealed. On the day of swarming recording was continuous.

of the day after the usual nocturnal cessation of the behaviour. In both cases well over 1,000 bees left the hive before the first DVAV was recorded. These observations do not agree with those of Allen⁵ on European bees, in which it seemed that DVAV among workers probably preceded the first flights of the day.

As maximal flight activity was associated with minimal DVAV frequency in the above examples, it was postulated that DVAV is a flight inhibitor and not a flight activator as suggested by Hamman and Allen⁴⁻⁶. These mutually exclusive hypotheses were tested as follows.

Allen has shown that the frequency with which a queen is vibrated rises to a peak on the day of swarming³. It might reasonably be predicted from the activation hypothesis that this frequency would remain high right up to the time of the queen's departure with the swarm, whereas the inhibition hypothesis predicts that it would have to drop sharply before the queen could fly. Figure 1 shows that during the last 5 h before departure of a swarm the DVAV frequency did, in fact, decline rapidly. Compared with the peak of 291 vibrations h^{-1} on the previous day there were only 25 during the last hour before swarming. Of these, only one occurred during the last 20 min. A similar result was obtained in a second trial and it agrees closely with a previously uninterpreted observation¹ that vibrations of the queen ceased altogether 30 min before a colony swarmed.

More direct evidence in favour of the inhibition hypothesis was obtained during a study of the behaviour of a virgin queen reared by a colony of about 1,500 bees. From the time of her emergence until the destruction of the last rival queen cell more than 8 h later this queen was not seen to be vibrated at all and she moved rapidly over the combs during the whole of this period except when being fed. She was first vibrated less than half an hour after the last queen cell had been attacked when, during a 6-min period, several bees vibrated her a total of twelve times. After the first three she slowed down considerably and after the next nine she stopped altogether for the first time. Thereafter, her activity diminished markedly (Table 1).

After repeating these observations on a second virgin queen with essentially similar results, it was concluded that DVAV is not exclusively a flight inhibitor, but a non specific activity inhibitor. It seems, furthermore, that one of its functions is the regulation of a queen's responses to the presence of queen cells. This latter conclusion was borne out by events recorded in two colonies that changed the direction of their behaviour from swarming to absconding.

Both colonies occupied two-frame observation hives and consisted of an estimated 4,500–5,000 bees. In the first, one sealed and two unsealed queen cells were discovered on December 17, 1974, but the colony was prevented from swarming by 4 consecutive days of overcast weather with intermittent rain and on day 5 the queen cells were destroyed. Figure 2 shows the frequency with which the queen was vibrated both during this

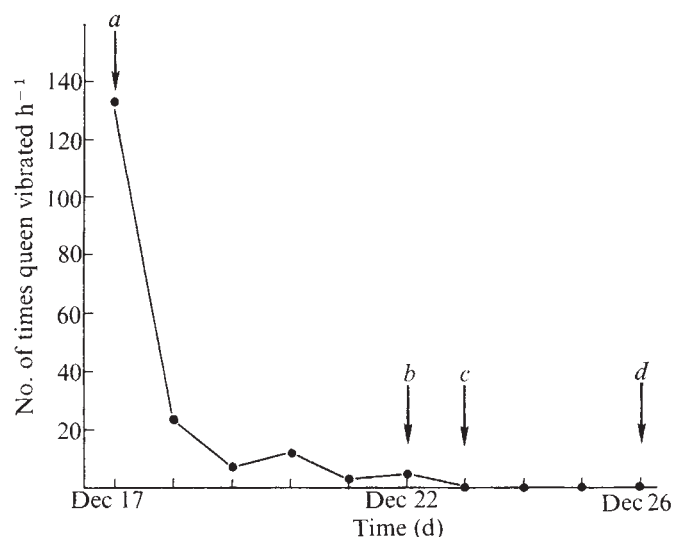


Fig. 2 Frequency with which a queen honey-bee was vibrated during a change in behaviour from swarming to absconding. *a*, Swarm cells discovered. *b*, Swarm cells destroyed. *c*, Colony attempted to abscond, but was prevented by a queen excluder at the hive entrance. *d*, Colony absconded after removal of the queen excluder.

Table 1 Relationship between DVAV, presence of rival queen cells and movement of a virgin queen

Observation period	No. of occupied queen cells	No. of times queen vibrated	Movement of queen	
Starting time	Duration (min)		Duration (min)	%
0530	30	4	30	100
0824	30	3	30	100
1005	15	3	15	100
1100	10	3	10	100
1200	10	2	10	100
1307	30	2	30	100
1416	30	0	24	80
1540	10	0	7	70
1605	10	0	3	30
1705	10	0	2	20

period and on the following days until the colony absconded. The destruction of the cells coincided with the virtual cessation of DVAV, but it was not observed whether the queen initiated the attacks on them. In the second colony, however, the queen was seen to attack two of the three sealed queen cells present following a sharp drop in the frequency with which she was vibrated. In this case the cause of the change in behaviour was unknown, the weather being favourable to swarming at the time.

In view of these observations, it seems that the response of a mated queen to the presence of swarm cells is very similar to that of a virgin to rival queen cells. She is strongly motivated to destroy them, but is prevented from doing so by a reduction in the level of her activity through the agency of DVAV. The observed increase in the frequency with which she is vibrated as the swarming cycle progresses probably reflects her increasing tendency to attack the swarm cells as their occupants mature.

Further evidence in favour of the inhibitory effects of DVAV on both queen and worker honey-bees, and the results of investigations into several of its functions will be reported in detail elsewhere.

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Occurrence of “pockets” of very small cells in adipose tissue of the guinea pig

THE concept of hyperplastic obesity, wherein the excessive number of cells in the adipose depots is thought to originate during overnutrition in early life, has evolved in the laboratories of Hirsch¹, Salans² and Björntorp³. The estimation of the number of adipose cells relies on (1) a measurement of total body fat and (2) a measure of the mean cell size. The two methods most commonly used for measurement of cell size are based on the microscopic examination of cells in a piece of fixed tissue⁴, or on sorting of cells in to size ranges by an electronic cell counter after dispersion of the tissue into a free cell suspension⁵.

An advantage of the microscopic method is that the disposition of cells within the tissue can be observed directly. In general it seems that cells of different sizes, whether they follow a normal or a polymodal distribution, are randomly distributed throughout the tissue. Nevertheless our studies have revealed a distinctly heterogeneous distribution of fat cells in different adipose tissues of the guinea pig. We shall describe the occurrence of isolated “pockets” or clusters of cells very much smaller than the general size range within the tissue.

In these studies the guinea pigs were of the Frant strain, 3 months old, fed *ad libitum* Oxoid modified diet 18, with cabbage twice a week.

For cell sizing, a modification of the microscopic method of Sjöström *et al.*⁴ was used. From each tissue site, four frozen sections (100 μ m) were cut and in each section 10 cell diameters were measured in one direction and then 10 in another direction perpendicular to the first. Each measurement was the maximum diameter obtainable by focusing through the section. The volume of each cell was calculated by computer making the assumption that adipocytes are spherical^{4,6}. No assumptions have been made about the distribution of the diameters, but the volume of each cell has been individually calculated and an average obtained from all the cells observed.

The volume of the adipocyte is essentially the volume of the triglyceride contained in it^{4,5}. The proportion of tissue occupied by adipocytes is represented by its triglyceride content, determined gravimetrically after ether extraction.

We have defined a pocket as a cluster of four or more cells distinctly smaller than the general size distribution within the tissue (Fig. 1a and b). The clusters, which are sometimes seen around blood vessels, have been observed in all the tissues examined (three subcutaneous sites: interscapular, anterior and posterior abdominal, and three deep sites: parametrial or epididymal fat pads and perirenal adipose tissue). The following discussion relates only to the frequency of pockets in fat taken from the posterior abdominal region of a female guinea pig. No attempt has been

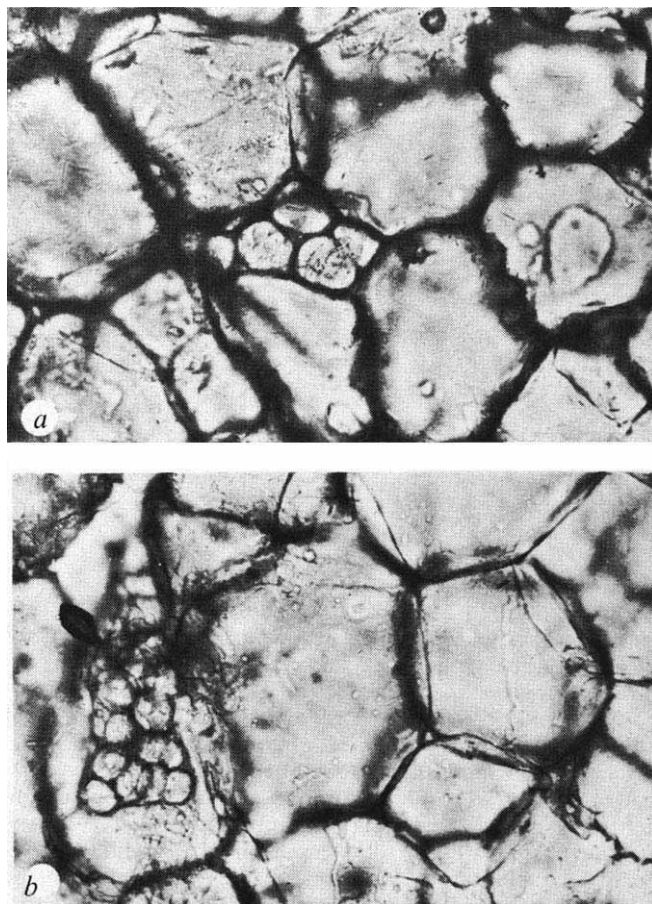


Fig. 1 a, Small pocket and surrounding “normal” cells.
b, Large pocket and surrounding “normal” cells.

made to quantify pocket frequency at other sites although qualitatively it seems to vary from site to site.

Pocket frequency was determined by examining 10 serial sections of 100 μ m thickness in a block of tissue measuring 6×11×1 mm (66 μ l). All cells in a section were scanned and all pockets noted. In this particular block there were 90 pockets containing an average of 5.67 cells per pocket (maximum 9, minimum 4). Therefore there were 510 pocket cells in the tissue block.

The mean cell diameter of the “pocket cells” was 59 ± 11 (s.d.) μ m (Fig. 2a) and of the “normal cells” 173 ± 31 (s.d.) μ m (Fig. 2b). These data correspond to a cell volume of 0.11 nl (0.10 μ g triglyceride per cell) for “pocket cells” and 2.72 nl (2.48 μ g triglyceride per cell) for “normal cells”. There is no overlap in the size ranges of the two population samples.

The triglyceride content of the tissue was $91 \pm 3.1\%$ ($\pm$ s.d. on six determinations). The volume of the tissue block occupied by fat cells was therefore 60.060 μ l.

The number of cells in the 66- μ l block of tissue is essentially the volume occupied by cells divided by the mean volume per cell. The presence of pocket cells complicates the calculation because of the difficulty of determining mean cell volume. The calculation has been done three ways.

(1) The volume occupied by “normal” cells is obtained by subtracting the volume occupied by 510 “pocket” cells from the total volume of tissue occupied by fat cells. The number of “normal” cells in this space is therefore 22,093 and the total number of cells is 22,603.

(2) If the observer recognises the existence of pocket cells but chooses to ignore them, the total cell number would be underestimated by 490 or 2.2%.

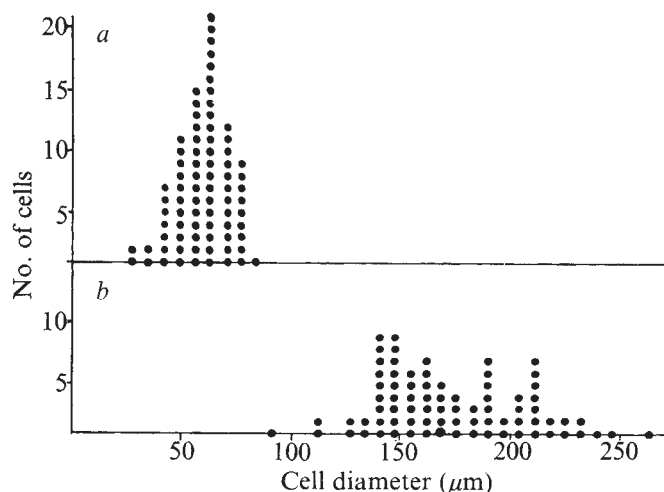


Fig. 2 *a*, Size distribution of "pocket" cells. *b* Size distribution of "normal" cells.

(3) If the observer includes a pocket of, say, four cells in his measurements, the calculated mean cell diameter would thereby be reduced and the total number of cells would be overestimated by 622 or 2.8%.

The results of this study reveal a distinct population of small adipocytes in the adipose depots of the guinea pig. Their mean volume per cell is only 4% that of "normal" cells and they represent only 2.3% of the cell population in terms of numbers and constitute only 0.09% of the volume occupied by adipocytes. The error introduced therefore, by inclusion or omission of these cells during the microscopic determination of cell size is small. The paper by Ashwell *et al.* which follows⁷ indicates, however, that the pocket phenomenon is more widespread than hitherto realised and we cannot rule out the possibility that in some tissues the frequency may be much higher rendering the microscopic method of determining mean cell size of dubious value.

But the results do highlight the desirability of microscopic examination of adipose tissue. Techniques based on sorting cells after digestion of the tissue would certainly reveal polymodality but would not distinguish between random occurrence of small cells throughout the tissue and the clustering of small cells in pockets as observed here. Moreover by detailed microscopy it may be possible to determine whether the pockets themselves are randomly distributed in the tissue, or whether their association with blood vessels is more than fortuitous. This raises the question of interpretation of the pockets in terms of tissue development. Preliminary observations suggest to us that pocket frequency decreases with age and this may indicate the pockets represent loci of most recent fat cell recruitment from a bed of pre-adipocytes. This possibility is being investigated by autoradiographic examination of tissue exposed to tritiated thymidine.

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Importance of fixed sections in the study of adipose tissue cellularity

IN the study of obesity in human subjects and animal models attention has recently been directed to the size and number of cells in adipose tissue. Measurements of fat cell sizes have led to the classification of hypertrophic, hyperplastic or mixed forms of obesity in animals¹ and man². A study of the literature concerning animals, however, reveals wide discrepancies in reports of the degree of hypertrophy or hyperplasia contributing to the expansion of fat mass observed in genetically^{3,4} or experimentally obese animals^{1,5-10}. These discrepancies have arisen not only because different species and strains of animals were used and different fat depots were sampled, but also because different methods were used to determine fat cell size and number.

Two of the most popular methods used for determining fat cell size are the osmium fixation of fat cells¹¹ and the sizing of isolated cells prepared by collagenase digestion^{12,13}. Both methods can be used to determine the distribution of cell sizes within a population and will yield a value for the mean cell diameter of the population. Neither method, however, gives any information about the *in situ* distribution of cell sizes or the

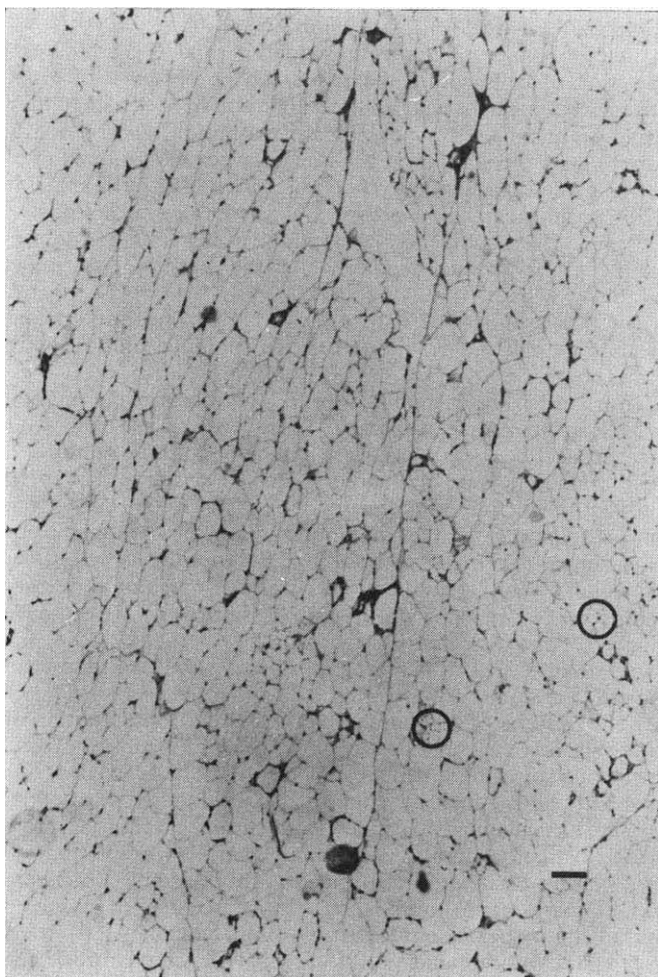


Fig. 1 Subcutaneous adipose tissue from a 4-month-old obese mouse. Fixed in Bouin's fixative and thin sections stained with haematoxylin and eosin. Scale marker, 100 µm.

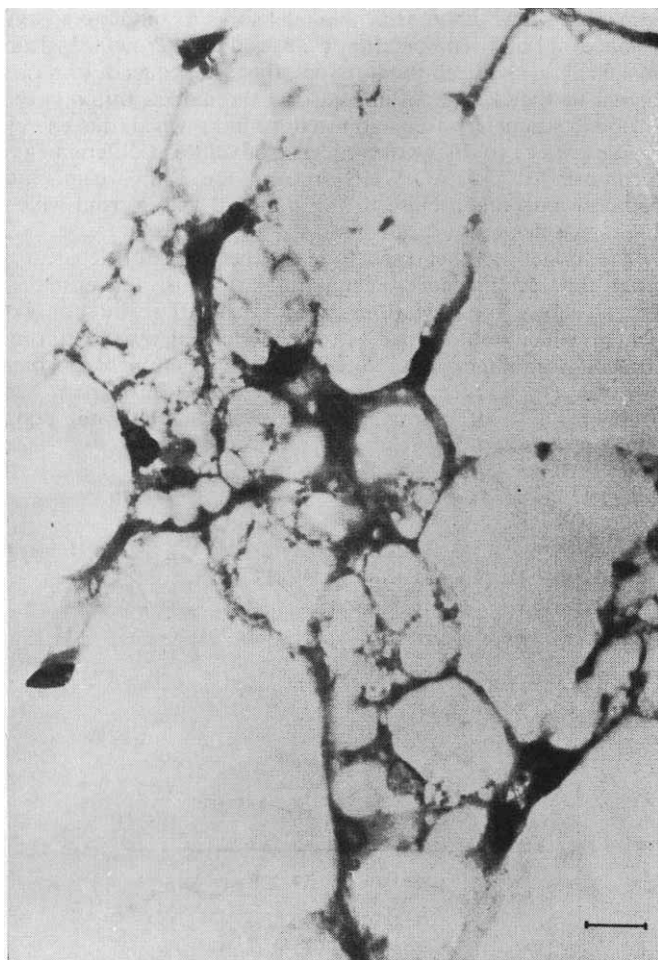


Fig. 2 Details as for Fig. 1. Scale marker, 10 μ m.

histological or pathological changes that might occur in the adipose tissue. We have studied numerous samples of adipose tissue from various sites of genetically obese mice and their lean littermates, and we propose that examination of fixed sections is essential if maximum information about adipose tissue cellularity is required.

Figure 1 shows a low power photograph of a fixed section of subcutaneous adipose tissue from a 4-month-old genetically obese mouse (C57BL/6J.ob/ob). The black circles show "pockets" of small cells which have developed between areas of larger cells. Figure 2 shows a high power photograph of one pocket. These pockets are particularly evident in the perirenal and subcutaneous fat depots and are not quite so obvious in the epididymal or parametrial depots. We have examined fat from obese mice from 4 to 40 weeks old and have seen pockets at all ages in obese mice.

A photograph with a final magnification of $\times 210$ showing the same field as Fig. 1 was used for sizing the fat cells. A representative area of large cells and pocket cells was chosen and this contained 153 cells. Cell diameters were measured with a ruler in two directions for each cell: the maximum diameter and the diameter perpendicular to this. The average of these two diameters was calculated and three determinations of mean cell diameter $\pm$ s.e.m. were made. For all cells, mean cell diameter was $70.5 \mu\text{m} \pm 2.96$; for normal cells, mean cell diameter was $89.2 \mu\text{m} \pm 2.66$; for pocket cells, mean cell diameter was $28.3 \mu\text{m} \pm 0.72$.

Several studies of adipose tissue development have concentrated on development of the gonadal fat pads (for example, ref. 14) probably because these are a very convenient source of tissue. Our observations on obese mice and also on normal rats have convinced us that the gonadal fat pads are unrepresentative

of other fat depots in animals and are certainly unrepresentative of human adipose tissue. Conclusions drawn from their study should be interpreted with caution. The photographs of fixed sections leave no doubt that pockets of small cells exist in some fat depots and it is tempting to speculate that these pockets represent loci for new fat cell formation. The development of adipose tissue has usually been studied in young growing animals and so far there is no general agreement as to the origin of fat cell precursors. Some investigators believe that fat cells arise from ordinary fibroblasts and others believe that they arise from special connective tissue cells, distinct from fibroblasts (see review in ref. 15). A detailed study of the location of these pockets of small cells should yield more information in this field.

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Pattern of fat and lean tissue deposition in children

THERE is evidence¹ of a prolonged change in body composition in children who have been overfed during the first year of life, and it has been suggested^{1,2} that there may be a critical period for fat deposition and adipocyte formation. We have examined published data on growth and body composition of normal children, and have found that these reveal cyclical changes in fat and lean tissue deposition which confirm the existence of critical periods, and offer an explanation for the observed changes in body composition.

From tables of average body weight extending from 13 weeks after conception³ and from birth to 17 yr (ref. 4), we have calculated increments in body weight per unit time (ΔM). Similarly, the increments in body fat mass (ΔF) can be calculated from data on average body composition at various ages^{3,5}. The difference between ΔM and ΔF at any given age is taken to be the change in lean tissue (ΔL). Figure 1 shows how the proportion of lean tissue deposited ($\Delta L/\Delta M$) varies with age throughout the growth period.

The changes shown are large and cyclical, and are unlikely to be artefacts in spite of the use of data from different sources for the pre- and postnatal periods. In early and midfoetal life, the deposited tissue is almost entirely lean, but fat deposition is favoured nearer full term. Throughout the first year of life, fat deposition predominates, but the proportion of lean rises to a maximum again at about 4 yr, when very little of the weight gained is deposited as fat. This cycle is repeated twice more,

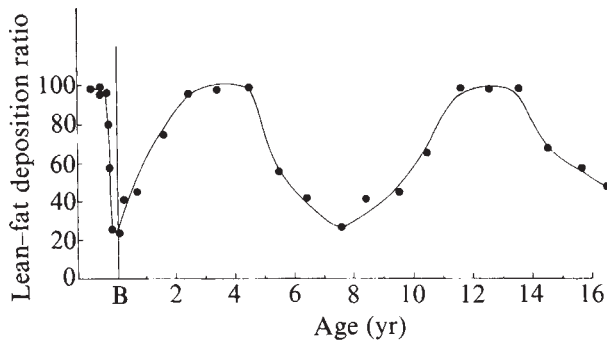


Fig. 1 Variations with age in the ratio between lean (ΔL) and fat (ΔF) deposition in normal males aged from 26 weeks after gestation to 16 yr.

with a return to predominantly fat deposition during the period between 6 and 10 yr, lean again between 12 and 14 yr (the period of the adolescent growth spurt), finally swinging back to fat deposition at 17 yr. In adults, changes in body composition consequent on experimental underfeeding, indicate that the lean-fat deposition ratio varies from about 50% for 'lean' types of individual down to 5% for 'obese' types.

In a computer model, which simulates weight maintenance in the adult, food intake energy is compared each day with energy expended for maintaining metabolic activity and for physical work (P.R.P. and A.E.D., unpublished). When positive, the difference is deposited as fat and lean tissue in proportions which are fixed for a particular physiological type of person (that is, tending to be 'lean' or 'obese'). If the daily energy balance is negative, sufficient tissues are mobilised to make up the deficit, but still in the same proportions. The model uses a random number generator to simulate the effects of daily variations in intake and expenditure, and predicts the time course of changes in body weight and composition that will result from feeding specified levels of daily energy intake.

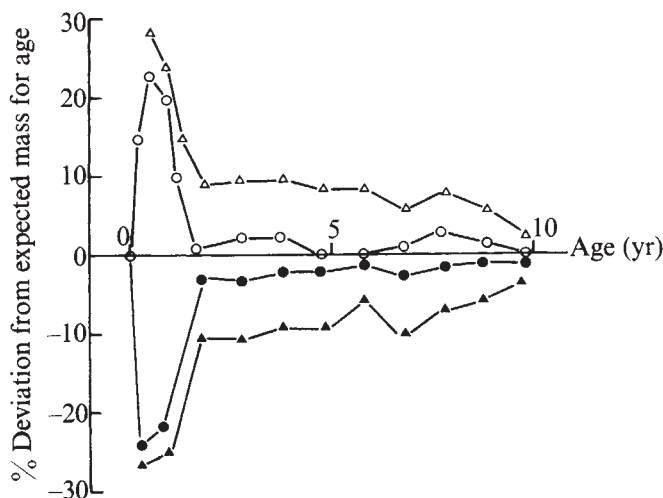


Fig. 2 Predicted changes in body mass in children who have been given a 20% deficit or excess in calories from birth to 1 yr and then a normal calorie intake. Changes are shown as percentage of expected mass for age. Δ and $\circ$, Fat mass and total body mass with excess calories. $\blacktriangle$ and $\bullet$, Fat and total body mass with a calorie deficit.

We have now developed the model so as to simulate energy balance and body composition changes during growth. Instead of a fixed ratio of lean to fat tissues, the time sequence of ratios shown in Fig. 1 is programmed into the model. Initially, the various parameters were adjusted so that when the energy intake is made to follow the sequence of values at different ages recommended by FAO/WHO (ref. 6), the body weight and composition remain close to the standard values from which Fig. 1 was derived.

The model was then used to study the effects of departures from the recommended energy intake. Thus, for example, Fig. 2 shows the deviations from expected weight and fat content which result from over- or underfeeding by 20% during the first year after birth. Note that the effects on body composition are marked, and that they persist throughout the following 9 yr of 'normal' feeding even though total body

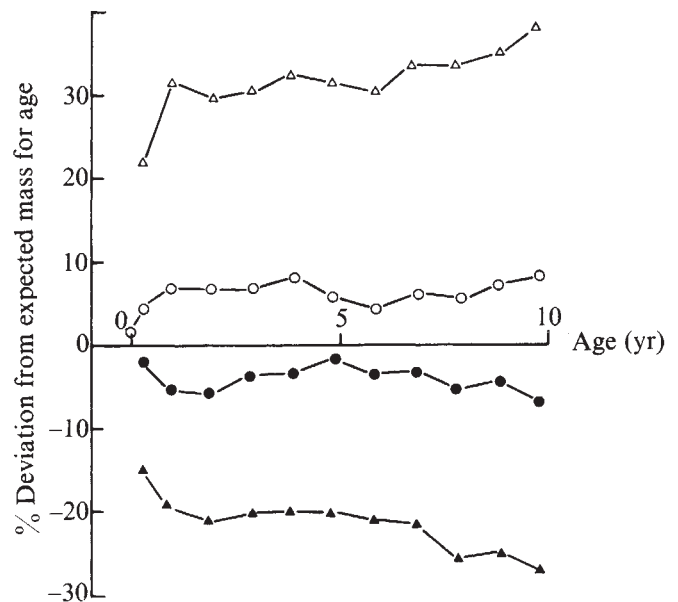


Fig. 3 Predicted deviations from expected mass for age when ratio of lean-fat tissue deposition is altered by $\pm 10\%$, and a normal calorie intake is given. Δ and $\circ$, Fat and total body masses with a 10% reduction in the lean-fat ratio. $\blacktriangle$ and $\bullet$, Fat and total body masses with a 10% increase in lean-fat ratio.

weight rapidly returns to near the expected values. This confirms clinical findings by emphasising the importance of over- or underfeeding during early life.

The programmed sequence of lean-fat tissue deposition ratios can also be changed. Thus, Fig. 3 shows the effects of changes $\pm 10\%$ imposed on all the values shown in Fig. 1, while maintaining energy intakes at the normal recommended levels. The upward shift produces a lean, ectomorphic individual; the downward, a plump endomorph. As has been mentioned above, there is experimental evidence that leanness or obesity in adults is associated with different values of the ratio $\Delta L/\Delta M$, and these studies support the idea that changes in the 'settings' of the ratio may explain many of the observed features of the development of ecto- and endomorphy in children. The metabolic mechanisms and the degree of genetic dependence of the ratios are unknown, but will clearly be important areas for future research.

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Eosinophils as mediators of antibody-dependent damage to schistosomula

WE have reported previously¹ that damage to schistosomula can be assayed by measuring release of ⁵¹Cr from labelled larvae. Using this technique, we have shown that schistosomula can be damaged by a combination of normal human peripheral blood leukocytes and of heat-inactivated sera from patients infected with *Schistosoma mansoni*¹. We now present evidence that the eosinophil is substantially the most active mediator of damage in normal peripheral blood, but that cells from eosinophilic patients are relatively inactive.

Schistosomula were prepared by allowing *S. mansoni* cercariae to penetrate mouse skin *in vitro*², and were stored overnight at 4 °C before labelling with ⁵¹Cr as previously described¹. Sera from five patients infected with *S. mansoni* were used as sources of anti-schistosomular antibody in different experiments. These sera had previously been tested for cell-dependent cytotoxic activity to schistosomula, and dilutions (1/24 to 1/90) were chosen which had given at least 50% cytotoxicity in the presence of unpurified leukocytes at an effector to target ratio of 2,000:1 over an 18-h incubation period. All sera were inactivated at 56 °C for 1 h before use, and were diluted in HEPES-buffered Eagle's minimal essential medium containing 10% heat-inactivated

Table 1 Reduction of cytotoxicity after pretreatment of effector cells with ANS or AES

Treatment	Complement	ANS + complement	AES + complement
Ratio of total effector cells to targets required for 30.5% isotope release	530	1,000	3,200
% reduction in cytotoxicity per added cell	0	47	83.5
% eosinophils killed	5	20	80
% neutrophils killed	5	100	10
*Ratio of viable eosinophils to targets in preparations giving 30.5% release	20	32	26
*Ratio of viable neutrophils to targets in preparations giving 30.5% release	302	0	1,728

*Based on a differential count in the complement control of 4% eosinophils, 60% neutrophils, and 36% mononuclear cells.

foetal calf serum (MEM/FCS). Effector cells from uninfected laboratory workers were prepared from heparinised blood by sedimentation of 5 volumes of blood with 1 volume of 4.5% dextran in phosphate-buffered saline for 30 min at 37 °C. Cells from the leukocyte-rich supernatant were washed twice in MEM/FCS, and were subjected to various purification procedures before testing for cytotoxicity. Eosinophil counts in the peripheral blood of the four normal subjects studied ranged from 90 to 240 mm⁻³. Unpurified leukocyte-rich preparations after dextran sedimentation contained 3–6% eosinophils. The cytotoxicity test was carried out in 7×38 mm sterile plastic tubes: each of four replicate tubes contained 0.1 ml of labelled schistosomula (500 ml⁻¹), 0.1 ml of diluted serum, and 0.1 ml of effector cells at various concentrations. After overnight incubation, half of the supernatant was withdrawn, and percentage isotope release was calculated from the count rates in both the pellet and the supernatant tubes. Statistical analyses were carried out on the logarithmically-transformed percentage isotope release data, using multifactorial analysis of variance and Duncan's multiple range tests³. Each of the experiments described here was repeated at least three times, with comparable results.

Titration of unpurified peripheral blood leukocytes for their ability to induce release of ⁵¹Cr from schistosomula in the presence of antibody revealed that isotope release was proportional to the logarithm of the effector to target ratio over a range of ratios from 50:1 to 5,000:1 (Fig. 1). The high ratios required presumably reflect the multicellular nature of the target organism.

Subsequent attempts to purify the effector cell showed that cytotoxic activity was progressively lost during repeated cycles of incubation with carbonyl iron and magnetic extraction. Although recent work⁴ has shown that some antibody-dependent effector cell activities in the mouse may be removed by incubation with uncoated but not with protein-coated carbonyl iron particles, this finding was initially taken to imply that the effector cell was phagocytic. It was then found that after centrifugation of the starting effector cell preparation over Ficoll-Hypaque⁵, the cells recovered from the pellet were approximately 10 times more active than those recovered from the interface (Fig. 2). For example, pellet cells induced 33% isotope release at a cell to target ratio of 90:1, whereas for the interface cells a ratio of 1,020:1 was required to induce the same level of release. The differential counts for the two cell preparations (legend to Fig. 2) show that the pellet preparation contained 88% polymorphonuclear cells (PMN) and 12% mononuclear cells (MN), whereas the interface contained 9% PMN and 91% MN. From these differential counts, it can be calculated that the ratio of PMN to targets in the preparation of pellet cells which gave 33% release

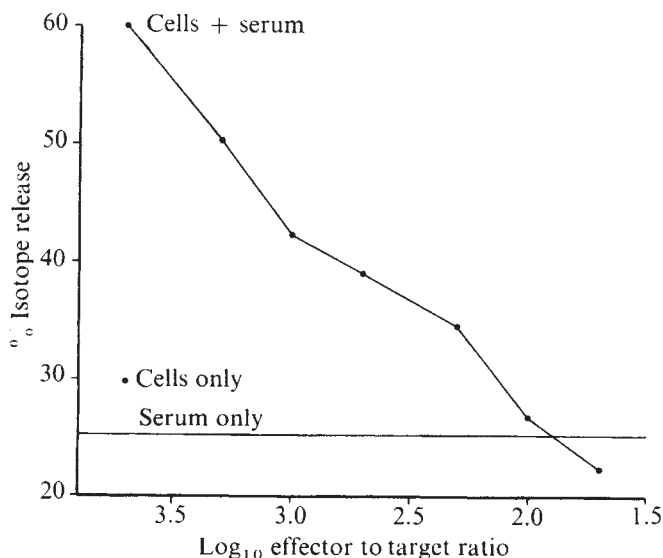


Fig. 1 Titration of effector activity of unpurified peripheral blood leukocytes, containing 62% neutrophils, 5% eosinophils and 33% mononuclear cells. Abscissa: log₁₀ ratio of unpurified effector cells to target schistosomula. Ordinate: percentage isotope release from schistosomula after 15 h incubation. Analysis of variance: "dilution" effect $P < 0.001$ ($F = 16.7.9$ and 30 degrees of freedom). Multiple range tests: "antibody alone" significantly less ($P < 0.05$) than 200:1 ratio and all higher ratios.

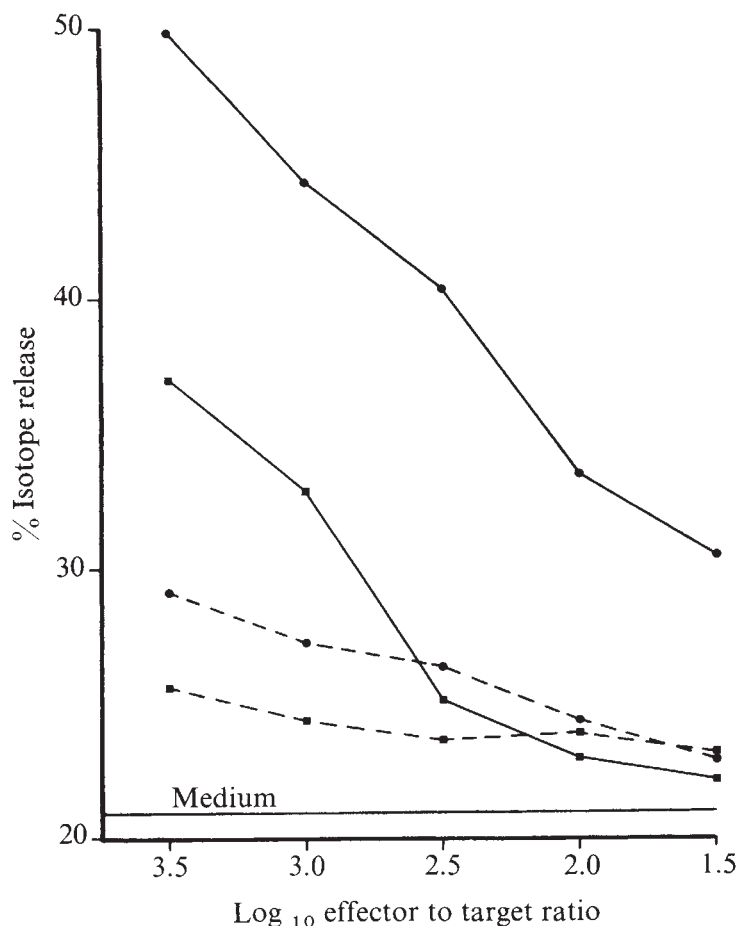


Fig. 2 Titration of effector activity of partially purified polymorphonuclear leukocytes and mononuclear cells. Cells from the leukocyte-rich supernatant of a heparinised, dextran-sedimented peripheral blood preparation were washed twice in MEM/FCS and overlayed on 10 ml of Ficoll-Hypaque⁵. This preparation was centrifuged at 400g for 40 min, and the interface layer and the pellet were washed twice in MEM/FCS before dilution and testing for cytotoxicity in the presence (—) or absence (---) of antibody. ●, Pellet cells, containing 8% eosinophils, 80% neutrophils and 12% mononuclear cells; ■, interface cells, containing 0.7% eosinophils, 8% neutrophils and 91% mononuclear cells. Analysis of variance: ("with antibody" only): "purification" effect $P < 0.001$ ($F = 100.6:1$ and 30 degrees of freedom). Interaction "purification" $\times$ "cell dilution" $P > 0.05$ ($F = 0.92:4$ and 30 degrees of freedom), indicating that the slopes of the titration curves are not significantly different.

was almost identical to the PMN to target ratio in the interface preparation which gave the same level of release (79:1 and 89:1 for the pellet and interface preparations respectively). In contrast, the ratios of MN to targets in these two preparations differed by two orders of magnitude (11:1 and 928:1 for the pellet and interface preparations respectively). Therefore, for a mononuclear cell to be responsible for damage, there would have to be a 100-fold selective purification of that particular mononuclear cell into the pellet. No evidence for this was available from other systems: it was therefore concluded that the active cell was a member of the PMN series. Since, however, both neutrophils and eosinophils were enriched to a comparable extent in the pellet (legend to Fig. 2), it was not possible at this stage to conclude which type of PMN was active.

The anti-human eosinophil serum (AES) and anti-human neutrophil serum (ANS)^{6,7} were then used in an attempt to identify the particular PMN involved. Unpurified effector cells were treated with AES or ANS and complement, and after subsequent washing were titrated for antibody-dependent cytotoxicity to schistosomula (Fig. 3 and Table

1). The AES and ANS reagents were also tested for direct cytotoxicity to purified neutrophils⁷ and eosinophils⁸ by Trypan blue exclusion (Table 1). The number of total effector cells in each preparation required to induce equivalent levels of isotope release was calculated from Fig. 3. Since the release induced by the AES-treated preparation was so low, the level chosen for comparison (30.5%) was that induced by the AES-treated sample at the highest cell concentration tested. From the total number of cells required, the reduction in cytotoxic activity per added cell could then be calculated for each preparation (Table 1). This reduction was related to the reduction in viable eosinophils, but not to the reduction in viable neutrophils. Equivalent levels of isotope release were induced by preparations containing comparable numbers of viable eosinophils, whereas there was no relationship between isotope release and the number of viable neutrophils (bottom two lines of Table 1). Since the starting preparation contained 4% eosinophils and 60% neutrophils, it could be calculated that each eosinophil was at least 27 times more active than each neutrophil in mediating cytotoxicity. Furthermore, the sum of the inhibitions induced by AES and ANS alone was greater than 100%, and part of the ANS effect may be artificial, since it may have been attributable to competitive inhibition of eosinophil cytotoxicity against antibody-coated schistosomula by the large numbers

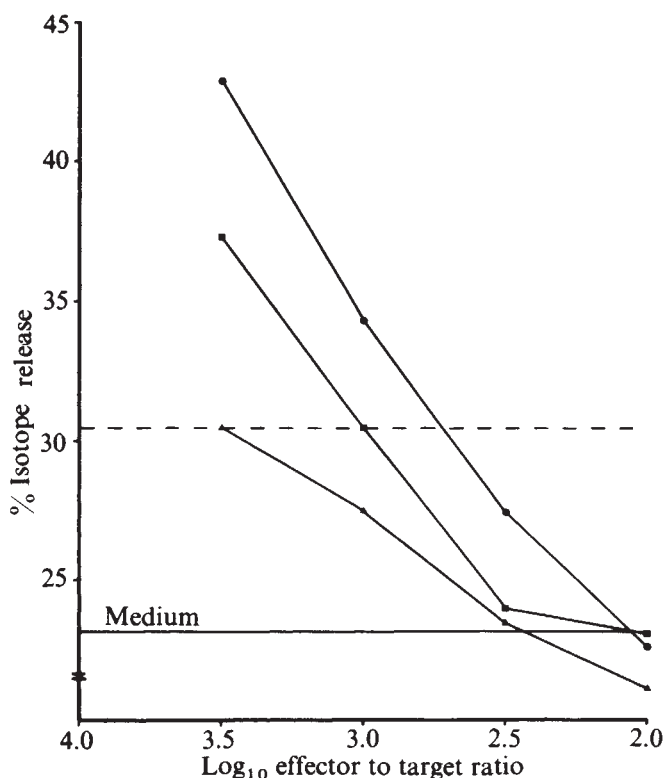


Fig. 3 Reduction of cytotoxicity after pretreatment of effector cells with AES and ANS. Unpurified leukocytes from a heparinised, dextran-sedimented peripheral blood preparation were washed twice in MEM/FCS. Aliquots were treated with MEM/FCS alone (●), with AES absorbed once with neutrophils and four times with erythrocytes, at a 1/10 final dilution (▲), or with ANS absorbed four times with erythrocytes, at a 1/10 final dilution (■). All preparations were incubated for 30 min and washed once with MEM/FCS. Fresh guinea pig serum (1/6 final dilution) was then added, and the preparations were incubated for a further 45 min at 37 °C before washing and testing for cytotoxicity to antibody-treated schistosomula. Analysis of variance: "antisera effect" $P < 0.001$ ($F = 22.2:2$ and 36 degrees of freedom). Interaction "antisera" $\times$ "cell dilution" $P > 0.05$ ($F = 2.35:6$ and 36 degrees of freedom), indicating that the slopes of the titration curves are not significantly different.

of antibody-coated neutrophils present in the preparation. This argument does not apply to the AES effect, since the numbers of antibody-coated eosinophils present in the AES-treated preparation would be too small to inhibit the much larger numbers of neutrophils. The inhibition induced by ANS therefore was more likely to be artificial than that induced by AES.

In contrast to these results, cells from three patients with marked eosinophilia (21% to 38%) induced by schistosomiasis or other helminth infections did not show the increase in cytotoxic activity which would be predicted if all eosinophils were equally active. One interpretation is that eosinophils from such patients may be blocked or inactivated by the immune complexes that elicited the eosinophilia. This possibility is now being tested.

These findings indicate that the major, and possibly the only, cell type in normal human peripheral blood capable of inducing antibody-dependent, complement-independent damage to schistosomula is the eosinophil. Other mechanisms for damaging schistosomula *in vitro* have been described^{2,9-11}, but the eosinophil-mediated reaction described here is potentially of particular importance in the light of subsequent findings (A.A.F.M., K. S. Warren and P. A. Peters, unpublished) that administration of AES abolishes resistance to reinfection in mice infected with *S. mansoni*. The observations described here, therefore, not only reflect a new eosinophil activity, but may also provide a useful model for analysing one mechanism of immune damage to schistosomula that does have relevance *in vivo*.

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Mediation of serum-induced changes in cyclic nucleotide levels in cultured fibroblasts by cyclic nucleotide phosphodiesterase

CELL cultures of untransformed fibroblasts can be maintained in a stationary or relatively quiescent state by adjusting serum concentrations to suboptimal levels¹. Addition of excess serum to quiescent cell cultures causes a rapid decrease in the intracellular concentration of cyclic AMP and a rapid increase in cyclic GMP levels²⁻⁵. These alterations in cyclic nucleotide concentrations precede the

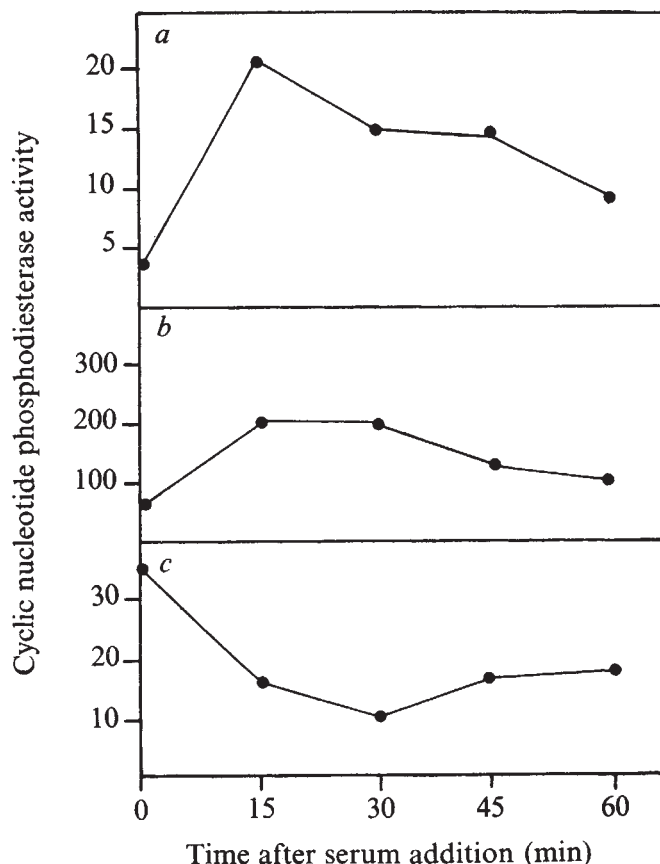


Fig. 1 BHK 21/c13 cells from surface cultures in 75 cm² Falcon tissue flasks were washed once with Eagle's minimal essential medium (MEM) containing 1% foetal calf serum. After trypsinisation (0.25% trypsin) cells suspensions were made in MEM and 1% foetal calf serum. 1×10^6 cells were seeded in 75 cm² Falcon culture dishes with 10 ml of MEM and 1% foetal calf serum and were incubated for 48 h at 37 °C. All cell cultures were then supplemented with an additional 10% foetal calf serum and incubated for the indicated times after which they were collected by scraping and centrifugation at 500g for 10 min. The cells were washed twice in Hanks' balanced salt solution; homogenised in 40 mM Tris (pH 8.0), and cyclic AMP and cyclic GMP phosphodiesterase activities determined according to the methods of Thompson and Appleman^{9,10}. Four culture dishes were pooled for each enzyme activity determination. The cyclic nucleotide activity is shown in pmol per mg protein per min. These data illustrate the results of a typical experiment. *a*, Cyclic AMP phosphodiesterase activity measured at a cyclic AMP concentration of 1.0 μM; *b*, cyclic AMP phosphodiesterase activity measured at a cyclic AMP concentration of 50 μM; *c*, cyclic GMP phosphodiesterase measured at a cyclic GMP concentration of 1.25 μM.

changes in macromolecular synthesis (such as RNA, protein and DNA synthesis) and growth induced by serum, and constitute one of the earliest steps in the sequence of events before the initiation of growth. The mechanism responsible for altering cyclic nucleotide concentrations in response to serum or other growth stimuli has not been elucidated however. We show here that a rapid alteration in cyclic nucleotide phosphodiesterase activity occurs when serum is added to quiescent fibroblasts and demonstrate that this alteration could mediate the changes in cyclic nucleotide levels.

Figure 1 illustrates the changes in phosphodiesterase activities seen after addition of 10% foetal calf serum to BHK 21/c13 (BHK cells, 1×10^6 cells per dish) cultured in media containing 1% foetal calf serum. Fig. 1*a* shows a 4–5-fold increase in cyclic AMP phosphodiesterase activity, measured at 1 μM cyclic AMP concentrations, within the first 15 min of serum addition. The total cyclic GMP

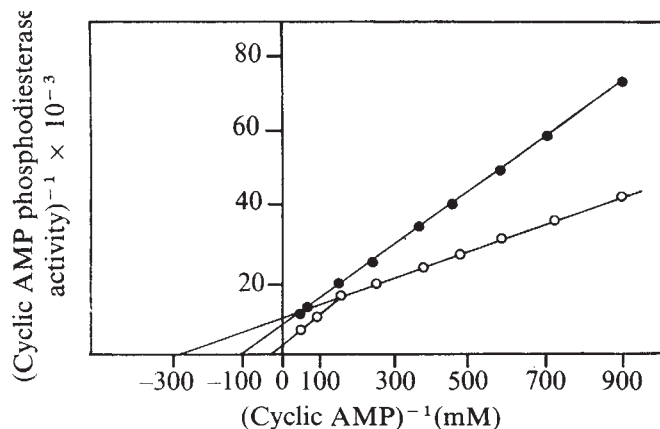


Fig. 2 Cells from ten culture dishes (1×10^6 cells per 75 cm^2 grown and collected as described in Fig. 1) were pooled, enzyme activities determined, and Lineweaver-Burk plots analysed. ●, Cells in 1% serum for 36 h; ○, cells that received 10% foetal calf serum for the final 30 min of incubation.

phosphodiesterase activity is shown to be reduced threefold at this time (Fig. 1c). Fig. 1b demonstrates an increase in total cyclic AMP phosphodiesterase activity after the addition of 10% serum. We have found that the cyclic AMP phosphodiesterase activity at zero time measured at $1 \mu\text{M}$ cyclic AMP may vary depending on the time of incubation in the 1% serum media and the serum lot used. An average increase of 3.5-fold (four experiments having a range of 2.8–5.3-fold increase) was seen, however, after addition of 10% serum.

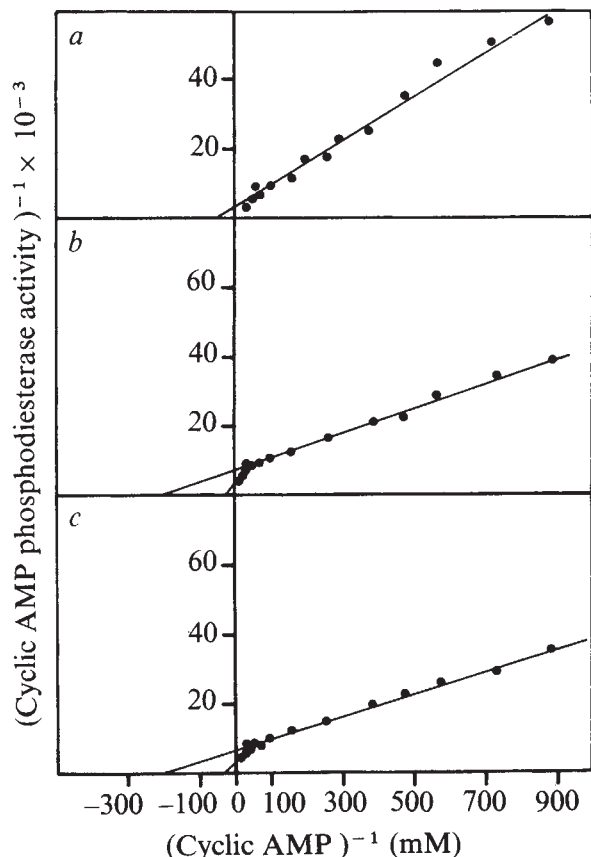


Fig. 3 Cells were prepared as described for the experiments in Fig. 2 with the exception that some cultures were incubated in cycloheximide (0.2 mM) for three hours before the addition of 10% serum. *a*, Cells in MEM with 1% serum and cycloheximide for the final 3 h incubation; *b*, additional 30 min incubation in 10% serum with no cycloheximide; *c*, same as in *a* except that after a 3 h preincubation in cycloheximide 10% serum was added to the cultures for the final 30 min.

Kinetic analysis of cell homogenates showed that the cyclic AMP phosphodiesterase of the cells incubated in medium containing 1% serum had an apparent K_m of $15 \mu\text{M}$ cyclic AMP (Fig. 2). Cyclic AMP phosphodiesterase activity from the cells which had been incubated for an additional 30 min in medium with 10% serum, however, showed activity with anomalous kinetic behaviour and apparent K_m values of 3 and $20 \mu\text{M}$ cyclic AMP. A 5-fold decrease in the K_m of cyclic AMP phosphodiesterase was evident within 30 min after the addition of serum to quiescent BHK cells.

To determine whether protein synthesis is required to obtain a serum-induced cyclic AMP phosphodiesterase

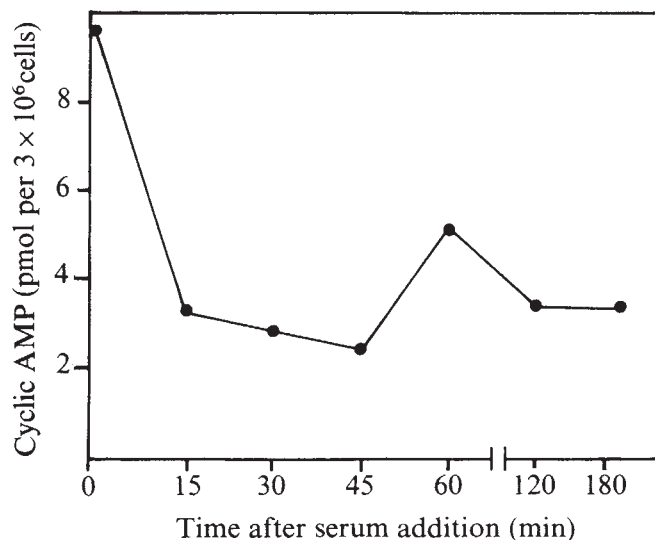


Fig. 4 Culture dishes (1×10^6 cells per plate) were incubated for 36 h. At zero time 10% serum was added to the plates and at the indicated times cells were collected for cyclic AMP determinations. To determine cyclic AMP content, the medium was poured from the plate and 0.05 N perchloric acid was added before scraping the cells from the plates. Cyclic nucleotide concentration was analysed by the radioimmunoassay according to Steiner *et al.*¹¹ as modified by Thompson and Williams¹².

activity with a greater apparent affinity for cyclic AMP, BHK cells were incubated as described for Fig. 2, except that during the 3 h before the addition of 10% serum either cycloheximide (0.2 mM) or a vehicle control was added. Following this treatment, cultures were either retained in 1% serum or had 10% serum added for the final 30 min incubation. The Lineweaver-Burk analysis⁶ of the homogenate activities from cells undergoing these treatments is shown in Fig. 3. The phosphodiesterase activities of the cells maintained in 1% serum in the presence of cycloheximide have a lower affinity (higher apparent K_m) for cyclic AMP than do the cells receiving 10% serum in the presence of cycloheximide (comparison of Fig. 3a with b and c). Analysis of the quiescent cells receiving additional serum did not reveal any differences in kinetic profiles between the vehicle control cells and those that received cycloheximide (Fig. 3b and c). These data indicate that the rapid change in substrate affinity of the cyclic AMP phosphodiesterase activity seen in quiescent cells in response to growth-promoting concentrations of serum takes place in the absence of protein synthesis.

Cyclic AMP levels decrease rapidly following the addition of 10% serum to quiescent BHK cells. The decrease in cyclic AMP levels is evident within 15 min after addition of serum (Fig. 4) and correlates with the increase in activity of cyclic AMP phosphodiesterase measured at $1 \mu\text{M}$ cyclic AMP (Fig. 1). Schröder and Plagemann reported that serum contains a cyclic nucleotide phosphodiesterase⁷. We also detected phosphodiesterase activity in foetal calf serum but

the data suggest little if any contribution of this enzyme to our experimental results. First, the specific activity of the serum enzyme using micromolar substrate concentrations of cyclic AMP in the assay is not sufficient to account for the changes in cyclic AMP phosphodiesterase activity seen in BHK cells after serum addition unless the cells did selectively extract all of the serum enzyme activity. Second, the serum enzyme can also hydrolyse cyclic GMP but we found a decrease in cyclic GMP phosphodiesterase activity in response to the addition of serum. Finally, previous studies with surface cultures of confluent BHK cells demonstrated a reduction in total phosphodiesterase activity when these cells were diluted to a lower density in 10% foetal calf serum⁸.

These data support our hypothesis that substrate affinities of the cyclic AMP phosphodiesterase activities are altered as a response to certain conditions that affect cell growth⁸. These same conditions result in a concomitant, rapid and transient change in intracellular cyclic nucleotide levels. Thus, the substrate affinity as well as the amount of phosphodiesterase could be involved in regulation of cyclic nucleotide concentrations. Alterations in substrate affinity of the enzyme may be an important early step preceding cyclic nucleotide changes and altered growth states.

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Evidence for immunoreactive somatostatin in the endocrine cells of human foetal pancreas

SOMATOSTATIN has been identified as a hypothalamic factor inhibiting the release of growth hormone^{1,2} by acting directly at the level of adenohypophyseal cells. It also acts on pancreatic endocrine cells^{3,4} to inhibit the secretion of glucagon and insulin. Immunohistochemistry has localised somatostatin in the median eminence^{5–7} as well as in discrete cells of the Langerhans islets of several species^{8,9}. The origin of pancreatic immunoreactive somatostatin poses a problem. It has been demonstrated that neither nerve fibres, nor nerve endings with immunoreactive somatostatin are found in the adult pancreas⁹. Therefore it has been suggested that the somatostatin detected in the pancreas does not

result from absorption by these cells of somatostatin synthesised elsewhere (in the central nervous system for instance) but is produced by the pancreatic cells (probably A1 cells) themselves⁹. The results reported here show the presence of immunoreactive somatostatin in the pancreas of normal human foetuses and one totally anencephalic human foetus.

The pancreases were removed from 6 to 24-week-old human foetuses obtained immediately after legal abortion, or from deceased premature infants. After fixation for 2 or 3 d in Bouin–Holland fluid, without acetic acid, and with 5% saturated Hg sublimate added, the tissues were carefully washed in water, then dehydrated and embedded in

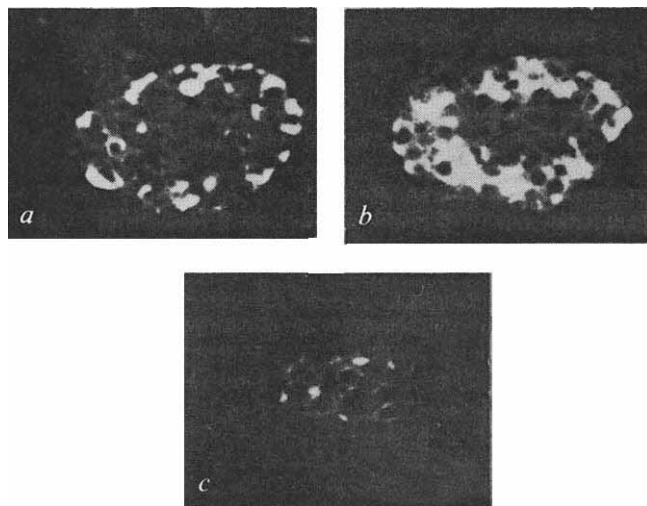


Fig. 1 Serial sections of a single islet of normal human 16-week-old foetus after application of a, anti-somatostatin; b, anti-glucagon; and c, anti-insulin.

paraffin. Sections (5 μ m) were used after fixing on to glass slides with 0.5% aqueous gelatin.

Hormones were detected in the cells using immunofluorescence. Anti-somatostatin⁹, anti-glucagon⁹ and anti-insulin were used as antisera. Immunofluorescence was detected by the indirect method with the aid of sheep anti-rabbit- γ -globulins conjugated with fluorescein isothiocyanate (Pasteur Institute, Paris). In all cases, the antisera were saturated with the particular proteins used in the coupling of antigen. The specificity of the immunofluorescent reaction was ascertained by studying the inhibition of the reaction by homologous and heterologous antigens (somatostatin, glucagon, insulin).

Figure 1 shows the reaction of the normal 16-week-old foetus endocrine cells with the three antisera. Three types of cells were distinguished—somatostatin-containing cells and glucagon-containing cells were often located in the islet periphery, insulin-containing cells, in the islet centre (Fig. 1a, b and c). Different cells reacted with each serum.

No immunofluorescent reaction was seen with anti-somatostatin and anti-glucagon before 10–11 weeks of gestational age and before 12 weeks of age with anti-insulin. At the third month, the fluorescent cells are located close by or in the wall of branching ducts embedded in connective tissue of the pancreatic parenchyma. After this time, they are located in islets.

Figure 2 shows the endocrine pancreatic cells of an anencephalic 7.5-month-old foetus reacting to anti-somatostatin. Immunofluorescence was also observed with anti-glucagon and anti-insulin. The cells were smaller than in the normal foetus. A fluorescent reaction was never observed

with anti-somatostatin either in nerve fibres or in nerve endings throughout the pancreas.

In the human foetal pancreas, inhibition of the immunofluorescent reaction was seen only after adding somatostatin in a concentration equal to $400 \mu\text{g ml}^{-1}$ of undiluted antiserum. Glucagon and insulin do not interfere with the immunocytological binding of somatostatin antiserum. Similarly, only insulin inhibits the immunofluorescence reaction caused by insulin antiserum, and only glucagon inhibits the immunofluorescence reaction caused by glucagon antiserum. No reaction was obtained using normal rabbit serum in place of the antisera.

It has been demonstrated and reported elsewhere^{7,9}, that the anti-somatostatin serum used here does not react with any of the following peptides—LH-RH, TSH-RH, oxytocine, lysine vasopressin, neurophysin, glucagon, insulin, secretin and the tetrapeptide Thr-Phe-Thr-Ser, common to somatostatin, glucagon and insulin. These data and the study of inhibition of the immunocytological reaction show that the fluorescence is most certainly attributable to the presence of somatostatin in the pancreatic cells. Therefore it



Fig. 2 Anencephalic human 7.5-month-old foetus pancreas. Cells containing immunoreactive somatostatin in two adjacent islets were detected by anti-somatostatin.

may be concluded that human foetal pancreas somatostatin occurs by 11 weeks of gestation.

The presence of somatostatin-containing cells in the islets of normal human foetal pancreas agrees with the recent observation of a somatostatin activity detected by bioassay in aqueous extract of foetal rat pancreas¹⁰. Also, the presence of glucagon-containing cells at 10–11 weeks of gestation and of insulin-containing cells at 12 weeks is consistent with the presence of the same hormone as demonstrated by radioimmunoassay for glucagon^{11,12} or insulin^{13,14} and by morphological techniques^{15,16}, all of these in human foetal pancreas. It has been suggested that the somatostatin-containing cells were the A1 cells⁹. Indeed, these cells have been identified in the human foetal pancreas from about the third month¹⁶.

Whether pancreatic somatostatin participates in some mechanism involved in the central control of glucagon and/or insulin secretion in the human pancreas during intra-uterine life is still unknown.

The data reported here demonstrate that somatostatin is not an exclusively hypothalamic hypophysiotrophic peptide and may be synthesised elsewhere.

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Scrapie incubation time can exceed natural lifespan

IN mice injected with mouse-passaged scrapie, the earliest rise in titre of agent is found in organs of the lymphoreticular system—irrespective of whether the injection is intracerebral or by a peripheral route^{1–4}. The titre of agent in the spleen, for example, can rise fairly quickly to a moderate plateau level and subsequently starts to increase progressively in the brain, where it reaches, during the clinical phase, a greater concentration than elsewhere. These generalisations are based on work in various laboratories with agent strain–mouse strain combinations having relatively short incubation periods in the range 150–300 d after injection of high doses of agent. Evidence of replication in the spleen is therefore an indication that the animal will eventually develop scrapie if it lives long enough.

The detailed titre levels and time sequences for the start of replication in the spleen depend on the strain and dose of agent, on the genotype of the mouse and its age at injection, and on the route of injection^{5,6}. In particular the gene *sinc* (alleles, s7 and p7) is important in controlling agent replication and the details vary widely for different strains of agent⁷. It has been shown with ME7 agent, that in mice homozygous for p7 there is a delay of about 4 weeks before replication starts in the spleen after injection of 10^4 C57BL intracerebral LD₅₀ units of agent whereas in *sinc*^{s7} mice the delay, if any, does not exceed 3 d; the respective incubation periods in these examples are approximately 300 d and 160 d.

Table 1 Number of infectious units of agent in spleen and incidence of clinical scrapie in C57BL mice injected intraperitoneally with various concentrations of 22A and, for comparison, incubation periods and incidences for the same doses given intracerebrally

Dilution of VM 22A-infected brain*	22A dose† injected	Intraperitoneal injection				Scrapie incidence	Intracerebral injection		
		60 d	22A concentration‡ per mg spleen at various intervals after injection	100 d	300 d	600 d	Incubation period (d±s.e.) or observation period (d)	Scrapie incidence	Incubation period (d±s.e.)
10 ⁻¹	20,000	ND	ND	ND	ND	7/7	562±15	4/4	452±2
10 ⁻²	800	0	0	25-50	25-50	0/4†	640-736	18/18	479±4
10 ⁻³	80	0	0	0	25-50	0/3	639-841	10/10	495±7
10 ⁻⁴	8	0	0	0	0	0/3	Discontinued at 630	4/6	548±13
10 ⁻⁵	≥1	0	0	0	0	0/4	ND	3/7	571±48
10 ⁻⁶	0	ND	ND	ND	ND	ND	ND	0/6	—

* 10⁻¹ inoculum, 500g, 10 min supernatant; others, 2,000g, 15 min, supernatant.

† VM intracerebral LD₅₀ units of 22A; estimated concentrations were within the ranges given.

‡ See text.

ND, Not determined.

For each agent strain-mouse strain combination, incubation period increases as dose is decreased and is longer after intraperitoneal than intracerebral injection. Even with intracerebral injections it is difficult to determine the titration endpoint for some agent-mouse combinations because the incubation period at the lower doses is of the same order as the lifespan of the mouse. Thus, with 22A agent in C57BL mice (*sine*⁸⁷) individual incubation periods range from about 450 d with 10⁻¹ 22A-infected brain homogenates to about 670 d with the 10⁻⁵ dilution, and many of the apparent survivors are senile by this stage (Table 1: estimated log₁₀ C57BL intracerebral LD₅₀ Reed-Muench titre is -4.6). In these senile mice there is an element of uncertainty in the histological criteria for deciding whether a mouse is dying of scrapie or of old age because there is an overlap in the range of histological lesions of scrapie and those caused by brain ageing⁸. Assay of agent in the spleen provides a potential means of resolving the dilemma of whether, in the type of example given, titre is being underestimated by cases of scrapie in the 'next higher dilution' not having the opportunity to appear during the normal lifetime. To assay this efficiently would require using another genotype of mouse in which the particular agent replicates much more rapidly, which in the case of 22A agent would be VM mice (*sine*⁸⁷). The following experiment shows that this approach is practicable in even more extreme circumstances.

C57BL mice were injected intraperitoneally at weaning with various saline dilutions of 22A-infected VM brain homogenate. One female and one male mouse in each dilution from 10⁻² to 10⁻⁵ was killed at 60, 100, 300 and 600 d after injection and the spleens removed for assay. The assay involved intracerebral injection of groups of weanling VM mice with 10⁻¹ C57BL spleen homogenates, which were not centrifuged, and estimating the titre from the incubation period⁹. The results for the female spleens are shown in Table 1; preliminary results (not given) for male spleens show the same general pattern.

In addition, some C57BL mice in each dilution group were observed for signs of scrapie: all the mice given the 10⁻¹ dose developed typical symptoms and lesions but in the lower dilutions none developed unequivocal signs. When individuals were senile or showing equivocal signs of chronic neurological illness they were killed and their brains examined histologically, as were the brains of all those killed earlier for spleen assay. None of the mice in the 10⁻² to 10⁻⁵ groups had typical scrapie lesions, but one of the four mice in the 10⁻² group (killed 709 d after injection) presented some difficulties in deciding that the lesions were probably caused by ageing—the spleen of this mouse had 50-100 VM intracerebral LD₅₀ units 22A mg⁻¹. The only other mouse brain in the whole experiment which presented such difficulties was from the 10⁻² group male killed at 600 d for spleen removal. It is possible therefore that scrapie was partly responsible for the equivocal symptoms and lesions seen

in a minority of the 10⁻² group. If only clinical cases of scrapie are used as the basis for calculation, the estimated intracerebral titre is 1,000 times higher than that for the intraperitoneal route but this reduces to about 50 times higher if allowance is made for animals with agent in the spleen.

The results of the spleen assays form a clear and consistent pattern, with agent only being detectable in the two higher-dose groups and being detected earlier in the 10⁻² group than the 10⁻³ group, but even then no agent was detected in the spleen until at least 100 d after injection. It is not yet known whether agent is present in the spleen for a few days immediately after injection and then cleared, but it seems unlikely that agent first recovered at 300 d is part of the original inoculum, slowly accumulating there. The reason for this long absence from the spleen is unknown and it could even be an artefact of insensitive assay systems. The crucial point will be whether agent can be detected elsewhere in the body during this interval and in which organs. Until these points are settled it would be quite inappropriate to refer to this interval as an 'eclipse phase' and without direct evidence it should not be assumed that this, or other, conventional virological categories apply to scrapie¹⁰.

If incubation can exceed lifespan with scrapie there seems to be no reason why this will not occur with other members of this group of diseases. It is therefore possible that a greater number of individuals are infected with one of the agents causing transmissible encephalopathies or dementias, such as Creutzfeldt-Jakob disease in man, than the number of cases which occur. Such individuals are, presumably, potential sources of infection, especially where tissue transplantation is involved¹¹.

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Translational control in the mealworm, *Tenebrio molitor*

ONE of the best known examples of translational control in a eukaryotic system comes from studies on the mealworm, *Tenebrio molitor*, in which control of the synthesis of adult cuticular proteins at the translational level has been proposed¹⁻⁷. From experiments using actinomycin D, it was concluded that the mRNAs for some of the adult cuticular proteins were synthesised by the first day of pupation¹. To translate these mRNAs *in vitro* on first day polysomes, however, the tRNA and enzyme from 6 to 7 d pupae were required²⁻⁴. Ilan *et al.* gave further evidence for a leucine tRNA which seemed to be controlling the translation of the mRNAs and the synthesis of which seemed to be in the control of juvenile hormone³⁻⁷. But our own studies of *Tenebrio*, reported here, lead us to question this translational control model.

We decided to look at changes of tRNAs during the development of *Tenebrio* and compare them with changes observed during the development of *Drosophila*⁸⁻¹⁰. Transfer RNA and aminoacyl-tRNA synthetases were extracted by the methods described previously⁸. Aminoacylation conditions were established that would give plateau levels of charging by 10 min. We found the conditions used by Ilan *et al.*⁴ for aminoacylating leucine tRNAs gave a very slow rate of charging chiefly because of the relatively low affinity of the *Tenebrio* leucyl-tRNA synthetase for leucine, the monovalent cation requirement and the ATP-Mg²⁺ ratio used. We found the K_m of the enzyme for leucine to be 20 μ M. This compares with an even higher K_m (200 μ M) reported by Ilan and Ilan⁷.

Using the improved aminoacylating conditions, the amounts of the leucine tRNAs as well as other tRNAs were determined in both early pupae and late pupae (pharate adults) (Table 1). When compared with either phenylalanine or tyrosine tRNAs, there was no significant change in the total acceptance of leucine by tRNA preparations from these two developmental stages. Even though there was not the expected net increase in the acceptance of leucine there could have been a new leucine tRNA formed with a concomitant decrease in the other isoaccepting species. To examine this possibility, ¹⁴C-leucyl-tRNAs from both early and late pupae were chromatographed on reversed-phase 5 columns by methods described previously⁸. The profiles of the leucyl-tRNAs from larvae, first-day pupae, last-day pupae and adults aminoacylated with a last-day pupal aminoacyl-tRNA synthetase preparation, were

virtually identical (Fig. 1). The same result was obtained using last-day pupal microsomal enzyme⁴ as well as larval, first-day pupal and adult aminoacyl-tRNA synthetase preparations. This was surprising in view of the results of Ilan and Ilan that indicated the synthesis of a new leucine tRNA^{5,7}.

We, therefore, went back to the conditions used by Ilan *et al.* for the aminoacylation of the leucine tRNAs (Fig. 2).

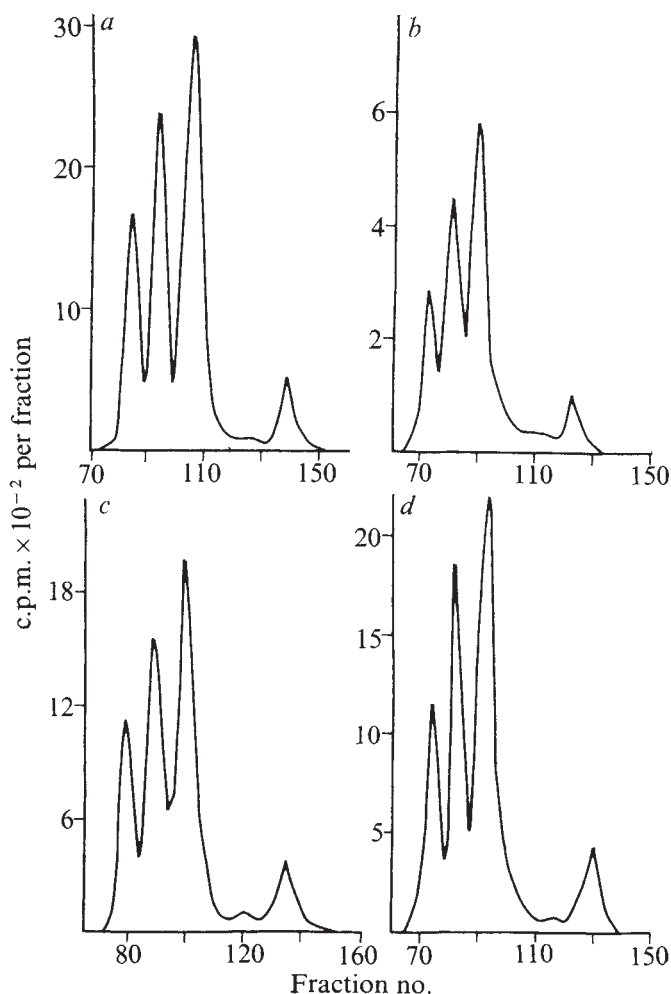


Fig. 1 Chromatography of ¹⁴C-leucyl-tRNAs from different developmental stages of *Tenebrio*. Aminoacylation was performed as described in the legend of Table 1 using last-day pupal aminoacyl-tRNA synthetase preparations. Chromatography of leucyl-tRNAs on RPC-5 columns was performed as described previously⁸. a, Larval; b, first-day pupal; c, last-day pupal; d, adult.

Table 1 Acceptance of amino acids by first-day and last-day pupal tRNAs

Amino acid	pmol amino acid accepted per A_{260} unit of total tRNA		First-day/last-day
	First-day pupal	Last-day pupal	
Leucine	55.3	51.5	1.07
Phenylalanine	26.3	25.4	1.02
Tyrosine	24.7	22.7	1.09

Transfer RNA and aminoacyl-tRNA synthetases were prepared as described previously⁸. Last-day pupal aminoacyl-tRNA synthetase was used. The aminoacylation conditions for leucine tRNAs were 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 8 mM ATP, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 50 μ M ¹⁴C-leucine, 1 mg ml⁻¹ enzyme preparation and 0.5 mg ml⁻¹ tRNA. The conditions for the aminoacylation of phenylalanine and tyrosine tRNAs were the same except 50 mM KCl, 4 mM ATP, 10 mM MgCl₂ and 25 μ M ¹⁴C amino acid were used.

The plateau level observed clearly represents incomplete charging and could not be used to estimate the amount of leucine tRNA present. We also chromatographed the ¹⁴C-leucyl-tRNA obtained in these conditions on RPC-5 columns (Fig. 3). It is apparent that peak 1 and peak 2 are poorly charged in relation to peak 3. Similar results were obtained when aminoacylation was performed in conditions where the amino acid or enzyme was limiting.

These results immediately suggested an explanation for the differences between our results and those of Ilan *et al.*³⁻⁷. As aminoacylation in suboptimal conditions can give rise to different isoacceptor profiles, any comparison of tRNAs from different developmental stages can only be performed when rapid complete charging is achieved. The charging conditions used by Ilan *et al.*⁴ produced rates that

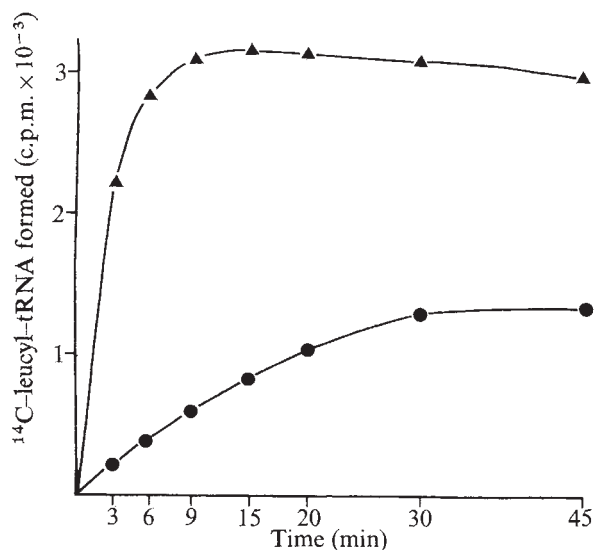


Fig. 2 Rates of aminoacylation of larval tRNA with leucine under varying conditions. ▲, Aminoacylation in the conditions described in the legend of Table 1; ●, aminoacylation conditions were similar to those described by Ilan *et al.*⁴; 50 mM imidazole, pH 7.0, 10 mM MgCl₂, 4 mM ATP, and 4 μM ¹⁴C-leucine.

gave plateau levels only after about 50 min, and the results presented here indicate that charging under such conditions is incomplete especially for certain leucine isoaccepting tRNAs. Another problem with any analysis of aminoacyl-tRNAs using dual labels^{5,7} is that the concentration of the differently labelled amino acids in both charging mixtures must be identical and the comparison made reciprocally to avoid isotope artefacts¹¹. This is especially true when the labelled amino acid substrates are used at concentrations that are well below the K_m value of the aminoacyl-tRNA synthetase. The observed differential charging rates could produce apparent control during the translation of mRNAs *in vitro*, due not to lack of a particular tRNA but to incomplete aminoacylation.

Another weakness in the evidence for translational control of adult cuticular protein relates to the amino acid

composition of these cuticular proteins. It has been claimed by Ilan *et al.*^{4,6}, apparently on the basis of published analyses on the beetle *Agrianome spinicollis*¹³, that adult *Tenebrio* cuticles are high in tyrosine (20%) whereas pupal cuticles have 'low tyrosine'. This difference was used as an index of the synthesis of adult cuticular protein *in vitro*⁴. It has been shown by Anderson *et al.*¹² that the adult cuticle has in fact less tyrosine (4.6%) than pupal cuticle (9%). This, therefore, invalidates the use of high tyrosine content as an index of adult cuticular protein synthesis.

The ability of actinomycin D to mimic the action of juvenile hormone suggests control at the transcriptional rather than the translational level¹⁴⁻¹⁶. Our results would support this in that we have found no evidence for a novel leucine tRNA that might control the translation of pre-formed mRNA for adult cuticular protein. We feel that the suboptimal conditions used to aminoacylate the leucine tRNAs, coupled with the differential rates of charging of the leucine isoacceptor tRNAs, explains much of the data that originally led Ilan *et al.*⁴ to suggest a translational control mechanism.

We thank Dr G. R. Wyatt for reading the manuscript. The work was supported by the National Research Council of Canada and the Rockefeller Foundation.

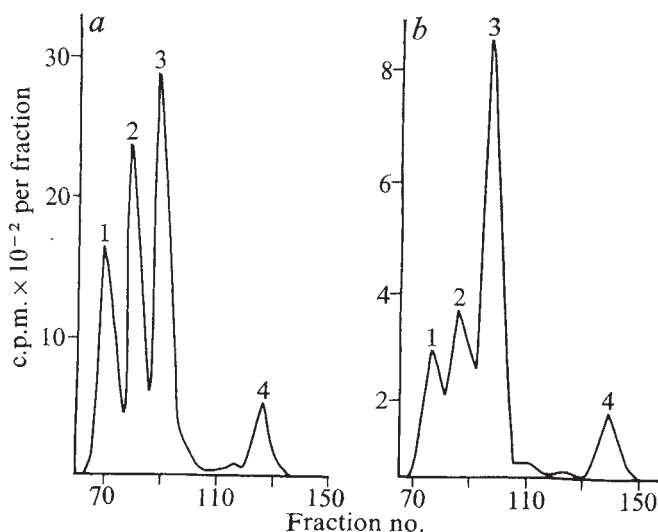
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Fig. 3 Chromatography of larval ¹⁴C-leucyl-tRNAs aminoacylated in different conditions. *a*, Aminoacylated in the conditions described in the legend of Table 1; *b*, aminoacylated in conditions similar to those used by Ilan *et al.*⁴, and described in the legend of Fig. 2.



Extraordinary pigment composition of a prokaryotic alga

WE report an anomalous unicellular alga with a prokaryotic cellular organisation like that of a blue-green alga, but with a pigment composition characteristic of green algae and higher plants.

Synechocystis didemni Lewin is a unicellular marine alga so far found only associated with colonial ascidians (*Didemnum* spp.) on the coasts of Baja California, Mexico¹. Under the light microscope it looks like a blue-green alga, and by electron microscopy of thin sections we have confirmed that the cellular organisation is prokaryotic, without membrane-limited nucleus, plastids or mitochondria (ref. 2 and R. A. L. and M. Schulz-Baldes, unpublished). Typically, blue-green algae—photosynthetic prokaryotes which can evolve oxygen when illuminated—contain at least one red or blue bilin pigment (phycocyanin or phycoerythrin) and only one chlorophyll, *a*, *S. didemni*, however, is leaf-green in colour, apparently

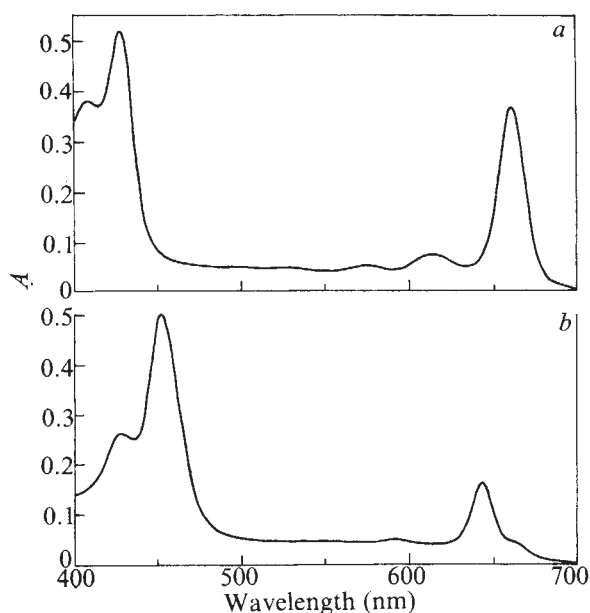


Fig. 1 Spectral absorption curves of solutions of *S. didemni* chlorophylls in ether, provisionally identified as *a* and *b*. For details of extraction and separation methods, see Table 2.

lacks bilin pigments, and contains two chlorophylls, *a* and *b*—features characteristic of green algae and land plants. Preliminary evidence for this unique combination of a prokaryotic organisation with a pigment complement so far only known in eukaryotic plants has been published elsewhere². We offer here confirmation of the presence of chlorophylls *a* and *b*, together with other lipid-soluble (but no water-soluble) pigments.

So far it has not proved possible to culture cells of *S. didemni*; consequently we had to analyse living material collected from the wild. In March 1975, didemnid colonies bearing *S. didemni* were gathered in a tropical lagoon on Isla S. José, Baja California from dimly illuminated mangrove roots (maximum light intensity around noon, 300–500 lx; water temperature about 20–25 °C). The surfaces were brushed as described², and the algal cells removed were concentrated by centrifugation. We collected three lots of algal cells, each of a few hundred milligrams (wet weight). The pigments from small subsamples were extracted and examined chromatographically on the day of collection; the rest of each sample was stored at –20 °C for careful examination in the laboratories of the Scripps Institution of Oceanography several days later. (There was no reason to believe that storage in this way effected any appreciable change in the pigment composition of the samples.)

After thawing, the cells were washed in water, and the supernatant, essentially colourless, was examined for possible traces of bilin pigments. An absorption spectrum revealed no

evidence for any pigmented material of this sort. The lipid-soluble pigments were then extracted and examined by thin-layer chromatography (TLC) and spectrophotometry (Fig. 1 and Tables 1 and 2).

The evidence for the existence of chlorophyll *b*, as well as *a*, in *S. didemni* seems unequivocal. This second green pigment cochromatographed with chlorophyll *b* from the chlorophyte *Codium fragile* and exhibited absorption maxima (Table 2) and band ratios typical for *b* (ref. 3). In the two samples examined the ratio *a*:*b* was found to be comparable with that of other algae which contain *b* (Table 1). The presence of *b* in our preparations could hardly be attributable to contamination of the samples by other algae, as microscopic examination revealed that the cells in our suspensions were almost all *S. didemni*, with no evident chlorophytes or euglenophytes. There was a small proportion (less than 1%) of diatoms or diatom debris, but these were mainly empty frustules, and only a few retained pigmented contents. The second chlorophyll of diatoms, *c*, has an *R_f* and other properties quite dissimilar from those of *b*. No *c* was detected in our material.

The predominant carotenoid in *S. didemni* is β carotene, representing some 65% of the total absorption attributable to yellow pigments. This proportion is higher than that normally encountered in chlorophytes (for example, 15% for *Chlorella pyrenoidosa*, which also has α carotene), but is close to that found in certain cyanophytes (for example, 46% for *Anacystis nidulans*⁵, 63% for *Microcoleus vaginatus*⁶). The other carotenoids of *S. didemni* were presumably xanthophylls, perhaps including myxoxanthophyll. The amounts were insufficient for critical analysis, which must evidently await the availability of much larger quantities of cell material, if and when this can be obtained in culture.

Although it has been generally accepted that all photosynthetic cyanophytes (blue-green algae) possess bilin pigments, in an unidentified blue-green alga associated with ascidians of the Great Barrier Reef little or no phycocyanin or phycoerythrin was detected⁷. It has also been generally accepted that blue-green algae lack *b* (ref. 8), although spectral data from blue-green algal mats collected in Yellowstone Park indicated that about 15% of the chlorophyll was present as *b* (ref. 9). Since those algae had grown at temperatures above 48 °C, they could hardly have been contaminated by chlorophytes or euglenophytes, none of which has been reported to grow in such hot water. Among the predominantly filamentous algae Inman⁹ noted the presence of *Synechococcus lividus* (Chroococcales) and of “a unicellular green alga, probably *Chlorella* sp.”. From what we now know of such habitats we can be almost certain that the latter alga was *Cyanidium caldarium*, which does not contain *b* (ref. 10).

In the original description of the type species of the genus, *Synechocystis aeruginosa*¹¹, the cells were described as being of a blue-green colour. Drouet and Dailly¹², who re-examined Sauvageau's material, reported that it contained cells which seemed to belong to the Chlorococcales (Chlorophyta); but it is hard to believe that Sauvageau could have mistaken a unicellular cyanophyte in this way. Further conclusions may only be drawn after examination of the pigments of an authentic

Table 1 Comparative pigment data for *S. didemni* and other algae

	Phycobilins (g per 10 ¹⁴ cells)	Chlorophylls			β carotene/ total carotenoids	Calculated from data in ref.
		<i>a</i> (g per 10 ¹⁴ cells)	<i>b</i> (g per 10 ¹⁴ cells)	<i>a</i> : <i>b</i>		
<i>S. didemni</i> (from white <i>Didemnum</i> *)		95	22	4.36	—	
<i>S. didemni</i> (from grey <i>Didemnum</i> *)		47	6.9	6.92	0.65	
<i>Anacystis nidulans</i>	1,300	60	0	—	0.46	5, 14, 15
<i>Chlorella pyrenoidosa</i>		40	7.6	5.25	0.15	4, 16, 17
<i>Euglena gracilis</i>		200	33.3	6.0	0.11	18, 19, 20

*The species could not be determined.

Cell counts were made with a Levy haemocytometer. Carotenoids and chlorophylls were estimated spectrophotometrically using standard extinction coefficients^{3,13}.

Table 2 R_f values and absorption maxima (nm) of *S. didemni* pigments

R_f on sucrose (TLC)	Colour	Solvent		Identity
		Acetone	Ethyl ether	
0.92	Yellow	425, 452, 480	—	β carotene*
0.72	Blue-green	430, 662	431, 662	Chlorophyll <i>a</i>
0.61	Orange	425, 451, 480	—	Unidentified xanthophyll
0.48	Yellow-green	457, 645	452, 642	Chlorophyll <i>b</i>
0.19	Yellow	440, 470	—	Unidentified xanthophyll
0.02	Orange	445	—	Unidentified xanthophyll

*Failed to separate from authentic β carotene (from *Daucus carota*) in a mixed chromatogram on $Al_2O_3 + MgO$ developed with 6% ethyl acetate in *n*-hexane²².

Pigments were extracted by sonicating the cells in a small volume of cold acetone. Cell residues were removed by centrifugation, and the pigments from the supernatant were transferred to ethyl ether by addition of cold, saturated saline. The ether extract was concentrated under N_2 and subjected to TLC on sucrose, using 1.3% *n*-propanol in ligroine (boiling point 63–75 °C)²¹.

strain of *S. aeruginosa* (we are attempting to obtain a culture of this species from the Department of Genetics, University of Moscow), and of the pigments of other cyanophytes grown in natural conditions, notably those encountered by *S. didemni* in mangrove swamps and by other species which survive in hot springs.

In conclusion, we have confirmed that natural collections of *S. didemni* contain chlorophylls *a* and *b*, β carotene, at least three xanthophylls, and no demonstrable water-soluble phycobilin pigment. In these respects it seems to be unique among cyanophytes which have been examined to date.

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Neuronal plasticity and recovery of function in a polyclad flatworm

NEURONAL recovery of function, although well known in the lower metazoa, has received comparatively little attention or critical investigation. We have been able to demonstrate not only recovery of function but also the recruitment of pathways mediating aspects of feeding behaviour in the marine polyclad flatworm, *Notoplana acticola*. Unlike some freshwater planarians which regenerate lost parts readily and may even grow new brains, polyclads have limited powers of regeneration and the brain seems to be essential for any repair which takes place¹. Three aspects of feeding behaviour were utilised in this study. When a normal intact flatworm is presented with food (dead adult brine shrimp) at its posterior lateral margin, that part of the

margin in contact with the food is extended and grips the shrimp. This local response is followed by an ipsilateral turning and contraction which brings the anterior margin in contact with the food which it grips and then passes the shrimp towards the midventral mouth. After feeding on two or three shrimp, the worms are satiated and no longer respond to the presence of food. Both turning and satiation are altered when the brain is removed. Decerebrate animals do not turn, the local response is prolonged, and the food is passed directly to the mouth where it is swallowed. After the stomach is filled the worms continue to collect food which is piled under the mouth. This localised feeding response (LFR) continues to occur as long as food is presented. The presence of the brain is necessary to inhibit the LFR as well as to initiate turning and satiation.

If a cut is made from the midline to the margin right through the entire body, that portion of the animal ipsilateral to and posterior to the cut will act as if it is decerebrate. The contralateral side, however, will still turn and with distension of the stomach this side will ignore offered food. A single animal can then be used for both experiment and control.

Table 1 Recovery of turning behaviour component of feeding in *N. acticola*, following a cut across one side of the body severing the nerve plexus

Treatment	Experimental side			Control side		
	T(%)	LFR(%)	N	T(%)	LFR(%)	N
Normal response	100.0	0.0	51	100.0	0.0	51
Cut (experimental side)	4.3	95.7	92	92.3	7.7	91
Healed	94.9	5.1	99	97.9	2.1	94
Cut (control side)	79.8	20.2	84	0.0	100.0	90

N, Number of observations; T, turning reflex.

Ten animals were used and, except for the initial test, approximately ten responses from each side were obtained. Sides were tested alternately. Animals were kept in small fingerbowls, 11 cm diameter and containing approximately 2.5 cm seawater. The water was changed every 2 d but the inner surface of the bowl was not wiped clean. This is critical as animals will not feed until a layer of slime has been deposited on the surface of the container². Dishes were not aerated and were maintained at ambient room temperature which fluctuated between 18 and 20 °C. With sufficient food animals can be kept indefinitely and will even grow. In each experiment 10 animals were used. Pairs were placed in separate dishes and allowed to acclimatise to the containers for 48 h before the start of the experiment. Individuals in a pair could be identified by size or slight colour differences.

Animals were tested for the turning response by being offered food on alternate sides (Table 1). Normal animals always turned to the side stimulated. During the course of the experiments described here food was always removed before it could be ingested. One side of each animal was cut (the experimental side) and the two sides were tested with food 20 h later. Nine of the ten animals now responded with LFR on the experimental side although one individual still turned in four of eleven tests. The control side turned in 92% of the tests and used LFR the other 8%. These animals were then retested 49 h after the cut had been made, by which time the edges seemed to have rejoined. When food was offered to the experimental side the animals turned 95% of the time. During these tests five LFRs were recorded from four of the animals. The recovery of turning behaviour

Table 2 Recovery and recruitment of feeding behaviour in *N. acticola*

Treatment	Experimental side			Control side		
	T(%)	LFR(%)	N	T(%)	LFR(%)	N
Normal response	100.0	0.0	100	—	—	—
Cut (experimental side)	5.6	94.4	84	100.0	0.0	91
Healed	92.1	7.9	98	97.9	1.1	98
Recut (experimental side)	72.1	27.8	103	100.0	0.0	86

Ten animals were used in these experiments, approximately ten readings were obtained from each animal.

could be accounted for in either of three ways. Functional connections could have been re-established across the lesioned zone. Secondly, new or unused pathways across to the contralateral side could have been recruited. Finally, mechanical coupling across the wounded area might have elicited activity from stretch or other sensory receptors which could then evoke the turning response. Histological examination showed that the severed nerves had become reconnected (Fig. 1). To see whether contralateral pathways were being recruited, the animals were cut across the control half slightly anterior to the previous cut on the experimental side. As might be expected the control side responded with LFRs to each food presentation. Eight of the animals turned toward food presented to the experimental side but two animals used LFRs consistently. The reappearance of LFRs suggested that in some cases recruitment of alternative pathways occurs. This was tested in a second series of experiments.

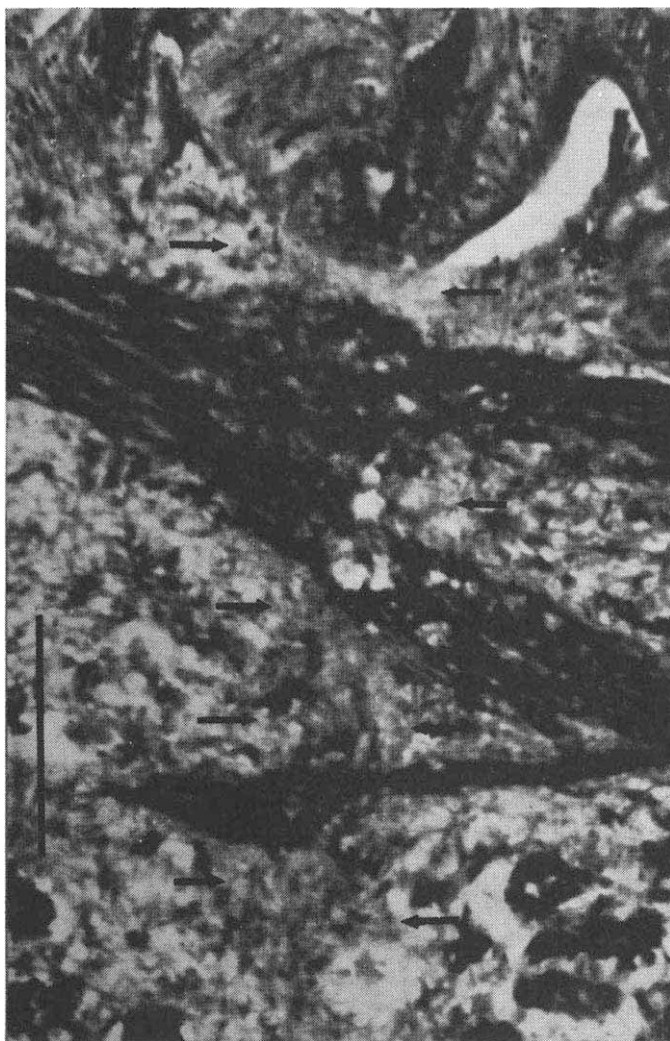


Fig. 1 Reconnected nerve trunks 49 h after lesion. Large nerve trunk running through the section had been cut at a branch point. Arrows are arranged on either side of the healed scar. Note apparent disorganisation within nerves across the scar. Bar, 200 μ m.

Ten animals were set out as before and tested for turning, which they all did. A cut was made across one side and they were tested for turning 19 h later. The experimental sides used LFR 94% of the time. Two of these animals did turn, one in two out of thirteen tests and the other in four of eleven tests. Table 2 shows that when the cut had healed 46 h later all the animals turned to the experimental side with only occasional LFRs. Once these readings were taken the original cuts were reopened and the animals retested 16 h later. Following the initial cut turning had only been recorded 5.6% of the time before healing but when the cut was reopened turning was recorded 72.1% of the time. The difference between these two results is statistically significant ($P < 0.001$, χ^2 test). All of the animals turned but seven also gave occasional LFRs. Turning behaviour to the reopened cut sides differed from that recorded after the cuts had healed. There was a considerable latency between contact and the initiation of turning which could last as long as 20 s in the reopened animals, whereas it was usually only 1–5 s in healed animals. The fact that animals still turned following reopening of the cut suggests that mechanical coupling is not a factor in turning elicitation. Histological examination of the reopened lesions indicated that in all but one case the nerves on the healed side had been completely resealed.

These results suggest quite strongly that functional connections can be made between severed portions of the nerve trunks and also that alternative, perhaps pre-existing, pathways can be recruited as well. Although such systems may have been suspected in these primitive systems previously, this seems to be the first time that such recovery of function has been clearly demonstrated.

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Influence of haemoglobin type on the induced ovulation rate in sheep

HARRIS and Warren¹ first reported haemoglobin (Hb) polymorphism in sheep; they separated a fast moving Hb A, and a slower moving Hb B, which were controlled by a single pair of alleles yielding three phenotypes—AA, BB and AB (ref 2). Several investigators have subsequently reported on the influence of Hb type on the reproductive performance of sheep. Evans and Turner³ found that Merino ewes of Hb types BB and AB were more fertile and had a greater proportion of multiple births than AA ewes; subsequent investigations have confirmed this observation⁴. In a study of Australian Merino ewes grazing oestrogenic clover pastures Obst *et al.*⁵ reported that AA and BB ewes were significantly more fertile than BB animals, suggesting that the A gene is associated with some factor which protects against the deleterious effects of plant oestrogens. We report observations in Indian Bikaneri ewes and suggest an association between Hb type and the ovarian response to exogenous gonadotrophins.

Seventy-eight adult multiparous Bikaneri ewes were classified according to their Hb phenotypes to give 12 AA, 36 BB and 30 AB animals. They were all kept in identical conditions of housing and management, and oestrus was detected by raddled vasectomised rams at 0900 daily; the day of onset of oestrus was classified as day 0. The BB and AB animals were subdivided into four groups and were injected with either 0, 500, 750 or 1,000 IU of pregnant mare serum gonadotrophin (PMSG,

Table 1 Influence of Hb type on ovarian response to PMSG in Bikaneri ewes

PMSG level (IU)		Mean number of ovulations ($\pm$ s.e.)							
		0		500		750		1,000	
Hb Type	<i>n</i>		<i>n</i>		<i>n</i>		<i>n</i>		<i>n</i>
AA	4	1.0		—		—	8	1.0 (nil)	
BB	6	1.0	10	1.6 $\pm$ 0.33 (3)	12	2.08 $\pm$ 0.077 (11)	8	3.5 $\pm$ 0.717 (7)	
AB	6	1.0	8	1.25 $\pm$ 0.070 (2)	8	3.0 $\pm$ 1.20 (6)	8	5.25 $\pm$ 2.20 (8)	

n, Number of observations.

Figures in parentheses indicate the number of animals which responded to PMSG treatment. Only those animals which had two or more corpora lutea in the ovaries were considered to have responded.

Gestyl, Organon) intramuscularly on the 13th day after oestrus. There were insufficient AA ewes to form more than two groups, which were either untreated or given 1,000 IU PMSG. The number of ovulations in response to this gonadotrophin treatment was assessed by counting the corpora lutea at laparotomy on the 5th to 7th day of the cycle following PMSG injection (Table 1).

None of the AA animals responded to the higher dose of PMSG, whereas there was a significant response ($P < 0.01$) of the AB and BB animals which was dose dependent (χ^2 test). The variation in the response of AA, BB and AB animals to 1,000 IU PMSG treatment was significant ($P < 0.01$) while between BB and AB animals was not significant (χ^2 test).

The ovulation rate of mammals is thought to depend primarily on the amount of gonadotrophins released into the blood⁶ rather than in changes in ovarian sensitivity to gonadotrophins⁷. The results of the present study strongly suggest that the number of follicles which will ovulate may also be determined by ovarian sensitivity. In analogous studies in mice, genetic differences in ovulation rate have been attributed to differences in ovarian sensitivity⁸⁻¹⁰.

Bindon *et al.*¹¹ reported that in a flock of Merino ewes selected for fecundity the ovarian response to a standard dose of PMSG was significantly higher in the group with the higher incidence of multiple births. Since response to PMSG is partly dependent on the endogenous gonadotrophin concentrations^{12,13}, a higher concentration of endogenous gonadotrophin in the BB or AB ewes may be responsible for the higher ovulation rate to PMSG. This possibility seems to be remote, however, since, irrespective of the Hb type, the incidence of twinning in the Bikaneri ewes is exceptional (2-3%). It may well be that the rate of secretion of endogenous gonadotrophin in BB and AB ewes may be lower than in AA ewes. Recent studies have revealed a significant variation in the peripheral plasma LH concentrations between ewes of same breed but of two different strains¹⁴. It also remains to be determined whether the sensitivity of BB and AB ewes to other hormones is also higher than that of AA ewes.

The results of this study suggest that haemoglobin type may provide a simple means of selecting sheep for increased sensitivity to gonadotrophins, and may also explain some of the variability in response to standard doses of gonadotrophin encountered in practice^{15,16}.

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Implication of absence of HCG-like gonadotrophin in the blastocyst for control of corpus luteum function in pregnant rabbit

IN the primate, the corpus luteum formed after ovulation has a relatively short life span. If the ovum becomes fertilised, however, the presence of the embryo in the uterus leads to 'rescue' of the corpus luteum and a prolongation of its functional life¹. Circumstantial evidence suggests that this is achieved through the elaboration of a luteotrophic substance (chorionic gonadotrophin) by the trophoblastic cells of the implanting embryo^{1,2}. Based on differences in the plasma progesterone levels of pregnant and pseudopregnant animals, a similar situation has been proposed for the rabbit³. In support of this, the blastocyst fluid from 6-d pregnant rabbits has been reported to contain a substance similar to human chorionic gonadotrophin (HCG), by a radioreceptor assay⁴, and a substance similar to luteinising hormone (LH), by a heterologous radioimmunoassay⁵. Our study was initiated to identify definitively the source of the HCG-like substance in the rabbit blastocyst. Using a highly sensitive *in vitro* bioassay method we have examined the concentration of HCG-like material in the serum throughout pregnancy and in the blastocyst from days 4 to 7 of pregnancy. We report here that the rabbit blastocyst does not contain or secrete any gonadotrophin which has significant HCG-like activity.

New Zealand White rabbits were mated and killed 4-6 d later. The uterine horns were removed and flushed from the oviducal end with physiological saline to expel the embryos, which were immediately transferred to incubation vials. The surrounding saline was aspirated and appropriate materials added for incubation. To obtain blastocyst fluid without loss of any critical material, the following procedure was adopted. Uterine horns removed on days 6 and 7 of pregnancy were wrapped in aluminium foil and frozen rapidly by immersing in an acetone-dry ice mixture. The uteri were then partially thawed, dissected longitudinally and the frozen blastocysts transferred to centrifuge tubes. After thawing, the blastocysts were ruptured, centrifuged and the supernatant obtained. The

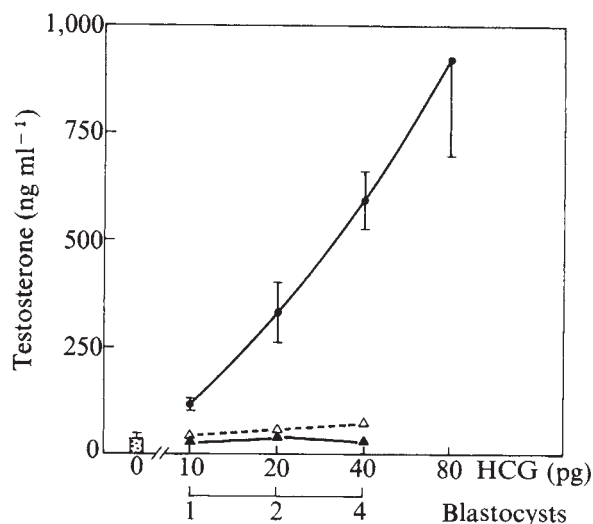


Fig. 1 Testosterone production by isolated mouse testes. Mice (70–80-d-old) were killed by cervical dislocation and the testes removed. After decapsulation the testes were rinsed with cold saline and placed in 16×50 mm glass vials containing 1 ml incubation medium (Krebs–Ringer bicarbonate buffer, pH 7.4, with 0.1% glucose) and graded doses of HCG or samples. HCG (10,600 IU mg^{-1}) was added in 100 μl incubation medium with 0.1% bovine serum albumin ($\bullet$). Using saline, blastocysts were flushed out from the uterine horns of 6-d-pregnant rabbits and immediately transferred to incubation vials. The blastocysts were either left intact ($\blacktriangle$) or punctured ($\triangle$) with a needle and the blastocoel fluid allowed to escape. All points were assayed in quadruplicate. Incubations were carried out at 34°C in a metabolic shaker at 150 cycles min^{-1} in an atmosphere of 95% O_2 –5% CO_2 . After 4 h incubation aliquots of incubation medium were diluted and stored at -20°C until the determination of testosterone by a direct radioimmunoassay⁶.

endometrial surface was scraped with a blade and the material coating the endometrium as well as part of the endometrium obtained. This was weighed, homogenised with 10 volumes saline, centrifuged and the supernatant obtained. The *in vitro* bioassay for HCG- and LH-like substances was based on the steroidogenic response of the mouse testes^{6,7}.

Figure 1 shows the testosterone production by decapsulated mouse testes. A dramatic increase in testosterone production was obtained with 20 pg of HCG (biological activity 10,600 IU mg^{-1}). When testes were incubated along with day 6 blastocysts, up to four freshly-obtained blastocysts did not stimulate testosterone production. This was the case for intact blastocysts as well as for blastocysts which were disrupted and the blastocoel fluid allowed to escape into the medium.

The steroidogenic response of a highly sensitive mouse Leydig cell preparation was used to evaluate the HCG-like activity (Fig. 2). Pregnant rabbit serum obtained 2 h after mating gave a dose-response curve parallel to that of HCG. In this system 200 μl day 7 blastocyst fluid or 100 μl of day 6 blastocyst fluid did not show any HCG-like activity. Saline extracts from 10 mg endometrial scrapings from 6- and 7-d-pregnant rabbits also showed no activity. To ascertain whether blastocysts secrete HCG-like material, embryos were flushed out of the uterus on day 4 of pregnancy and preincubated for 21 h. Leydig cells were then added and incubation continued for a further 3 h. No evidence for the secretion of HCG-like material was noted.

The Leydig cell preparation was used to measure HCG-like activity in the rabbit serum throughout pregnancy. By 0.5 h after coitus there was a 100-fold increase in the HCG-like activity (Fig. 3). After reaching maximum levels at 2 h *post coitum* the activity declined by 4 h and had returned to pre-mating levels by 24 h. The levels remained low throughout pregnancy and did not rise above the pre-mating levels. The recovery of HCG added to sera or blastocysts was quantitative.

These results demonstrate that coitus is followed by a rapid increase in the serum HCG-like activity which reaches a peak in 1 to 2 h and then decreases quickly to pre-mating levels, at which it remains throughout pregnancy. The post-coital LH levels measured by radioimmunoassay show a similar pattern⁸. No significant HCG-like activity could be detected in the blastocysts between days 4 and 7 of pregnancy. Haour and Saxena⁴, using a radioreceptor assay, reported that on day 6 of pregnancy the concentration of HCG-like material in the blastocoel fluid was tenfold higher (87 ng ml^{-1}) than that in the serum (6–8 ng ml^{-1}). They also reported that the plasma levels of HCG-like material in the 6-d-pregnant rabbit was significantly higher than that in the non-pregnant animal. Results of the present study which are based on a highly sensitive *in vitro* bioassay do not confirm the results obtained by radioreceptor assay. This discrepancy is probably related to the different methods used. Comparison of the radioreceptor assay with radioimmunoassay and *in vitro* bioassay has shown that the radioreceptor assay was subject to nonspecific interference by plasma proteins⁹.

In the rabbit, a reflex ovulator, mating induces ovulation and corpus luteum formation. If the ova are not fertilised a pseudopregnancy of 17 d duration follows. The corpus luteum of pseudopregnancy shows regressive changes by day 12. Most of the evidence suggests that the non-pregnant rabbit uterus produces a luteolytic substance, presumably a prostaglandin, which limits the duration of corpus luteum function¹⁰. The biological evidence for the existence of a luteotrophic substance derived from the conceptus before day 10 of pregnancy is equivocal. Using a competitive protein-binding assay plasma progesterone levels were reported to be significantly higher in pregnant rabbits during the preimplantation stages than in pseudopregnant animals at similar stages³. A radioimmunoassay, however, showed no such differences¹¹.

The significance of the detection of HCG-like substance by indirect immunofluorescence on the surface of mouse embryos

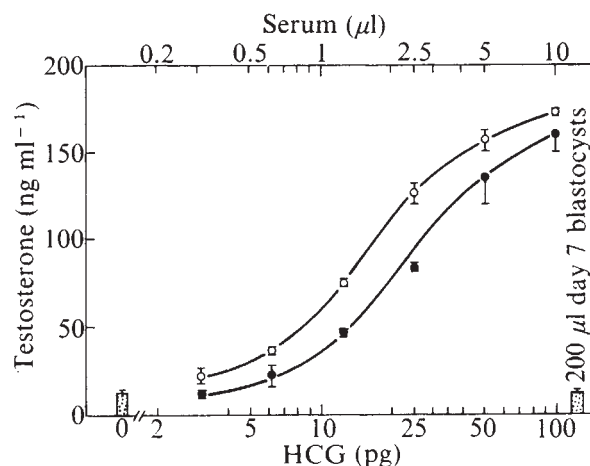


Fig. 2 Testosterone production by mouse Leydig cell preparation. Four mice were killed by cervical dislocation and the testes removed. After decapsulation they were rinsed with saline and placed in a beaker with 25 ml medium 199 (Difco) containing 2% foetal bovine serum. Testes were chopped into small pieces with scissors and stirred for 10 min at room temperature on a magnetic stirrer. The medium was then filtered through a fine nylon mesh and centrifuged at 600g for 15 min at 4°C . The supernatant was discarded and the cells resuspended in 25 ml medium. A portion (0.25 ml) of the cell suspension was added to tubes containing 0.1 ml standards or samples. HCG ($\bullet$) was dissolved in the incubation medium. Serum obtained 2 h *post coitum* ($\circ$) from two rabbits was pooled and diluted with incubation medium. Blastocyst fluid (vertical bar) from 7-d-pregnant rabbit was lyophilised and reconstituted in the incubation medium to half its original volume. The rest of the procedure was as in Fig. 1 except that the incubation time was reduced to 3 h; stippled column, control.

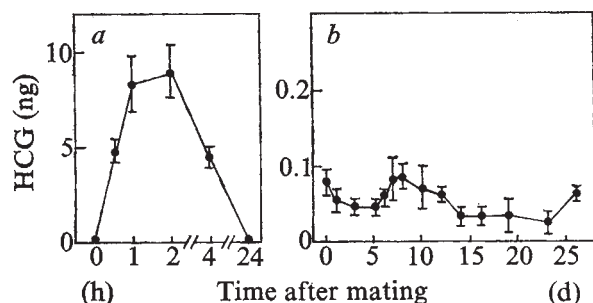


Fig. 3 Serum levels of HCG-like material in the pregnant rabbit measured as in Fig. 2. Blood samples (3–4 ml) from the marginal ear vein were collected before mating, at frequent intervals after mating and at less frequent intervals throughout pregnancy. The high HCG activity at 1 and 2 h post coitum (a) represents the presumptive LH peak. The levels are much lower throughout pregnancy (b, note change in scale).

during the morula stage but not during the blastocyst stage is not clear¹². A role for HCG in the protection of the foetus against attack by the maternal immunological system has been suggested¹³. Perhaps substances resembling HCG reported to be present in the unimplanted mouse and rabbit embryos play a role in the immunological system but are not gonadotrophins.

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T-T interaction in the generation of helper cells *in vitro*

THE interaction of different cell types is now widely recognised as being of fundamental importance in the generation of many different immune responses^{1,2}, and occurs in many species. Several broad groups of interactions have been described—between macrophages and lymphocytes^{3,4}, between T cells, B cells (and macrophages) in antibody production^{1,3,5} and between subpopulations of T cells in the graft versus host responses⁶, and in the generation of cytotoxic T cells^{7–10}.

Levy and her colleagues have reported that the antigen performic acid oxidised ferredoxin (OFd), derived from *Clostridium pasteurianum* has only 2 major antigenic determinants located in the carboxyl and amino terminal regions of the molecule¹¹. They reported that cooperation between cells primed to the determinants situated at the COOH- (C-hapten) and at the NH₂-terminus (N-hapten) is necessary for the generation of a proliferative response of guinea pig lymph node cells *in vitro* to the whole antigen, OFd^{12,13}, but not for the expression of hypersensitivity reactions. Having recently described a system for the development of helper cells *in vitro*¹⁴, we wanted to

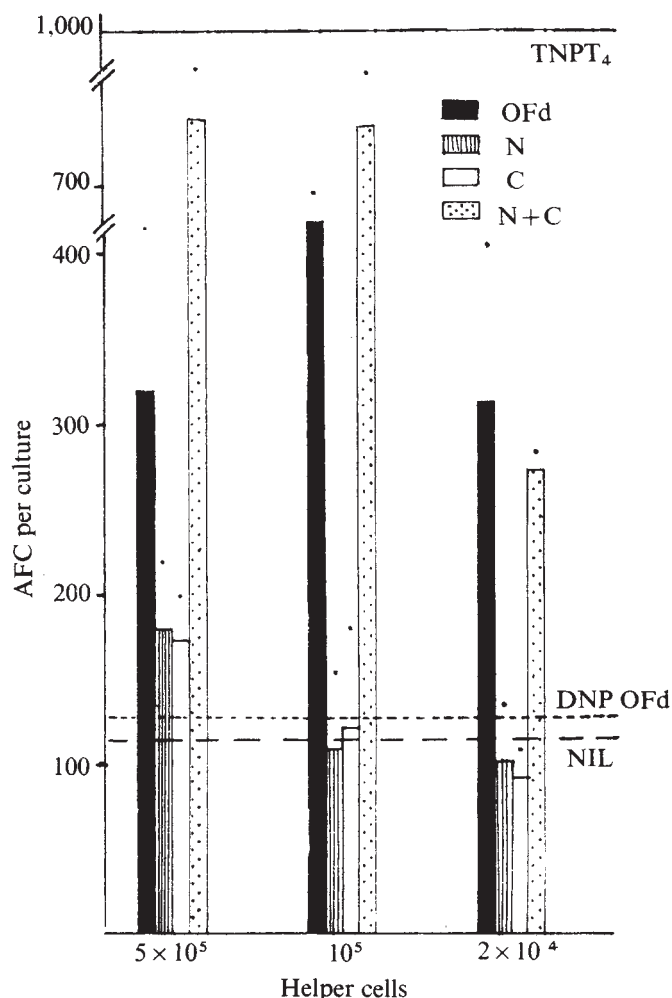


Fig. 1 Synergy between N and C primed T cells. Spleen cells were treated with a cytotoxic rabbit anti-mouse B cell antiserum, prepared by repeated injection (3 or 4) of anti-θ treated spleen cells which had been depleted of dead cells²² and red cells²³. About 10⁸ cells were injected at 2-weekly intervals, half intramuscularly emulsified in Freund's, and the rest intravenously. B cell contamination after treatment with the antiserum and complement ranged from 0–3% in replicates. Significant helper cells to OFd were only induced provided OFd primed T cells, or a mixture of N and C-BSA primed spleen cells were used. The dots above the bars indicate the upper limit of the standard error.

determine whether the T helper cell response to OFd required cooperation between two populations of T cells, in analogy with other cooperative proliferative response to OFd¹¹.

By using the chemically defined determinants of OFd either linked together as in the whole antigen, or separately, coupled to bovine serum albumin (BSA), it was possible to prime cell populations *in vivo* to both or to either of the individual determinants and subsequently to use these cells *in vitro* to test for cell interaction during the development of helper cells to OFd. Cells were initially cultured for 4 d in the presence of OFd to generate helper cells directed towards OFd, and then small numbers of these cells were transferred to a second culture where, in the presence of excess B cells and macrophages and with dinitrophenylated-OFd (DNP-OFd) helper cells directed towards OFd would augment the plaque forming cell response to DNP. Here we describe the use of three distinct experimental approaches which have previously been used to study T-B interactions. They demonstrated that the interaction of two T-cell subpopulations is essential for the development of helper cells to OFd *in vitro*.

The first approach was the classical one of synergy, which

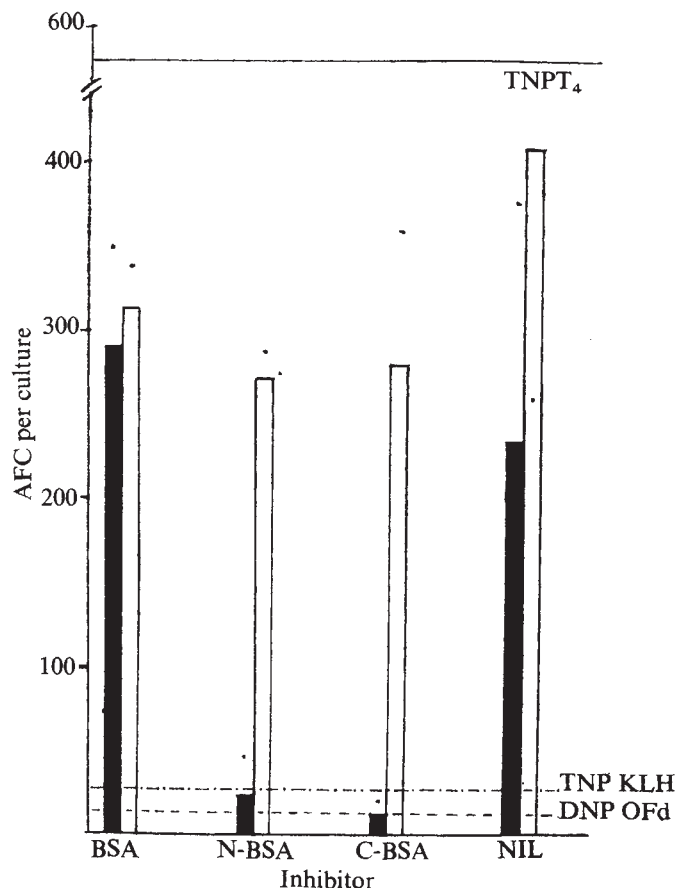


Fig. 2 Effect of excess N or C hapten on the response to OFd. Either bovine serum albumin (BSA) or N-BSA or C-BSA was added to cultures of OFd primed T cells containing OFd ($1 \mu\text{g ml}^{-1}$) and KLH ($0.1 \mu\text{g ml}^{-1}$). N-BSA, C-BSA or N or C-S-BSA (made with succinylated BSA) and N or C poly-D-glutamic acid (PDG) all suppressed the helper response to OFd. Substitution ratios of the preparations used ranged from 10–20 N or C groups mol^{-1} . These were made as described previously¹². All these compounds were toxic in high concentrations, with non-specific diminution of the KLH response beginning at $30 \mu\text{g ml}^{-1}$ of N or C-PDG, and $100 \mu\text{g ml}^{-1}$ of N or C-BSA, or succinylated BSA. The inhibition results shown were with $10 \mu\text{g ml}^{-1}$ of N or C-BSA. Inhibition was also found with $1 \mu\text{g ml}^{-1}$ of N or C-BSA, but not with $0.1 \mu\text{g ml}^{-1}$. Dot above the bar graph indicates the standard error. Dark bar represents the response to DNP OFd, the unshaded bar represents the response of the same cultures to TNP KLH.

with anti- θ antisera verified that the helper cells were, as usual, T cells (data not shown).

Inhibition of cooperative responses by unconjugated carrier or hapten has been reported both *in vitro*¹⁶ and *in vivo*¹⁵. Responses that do not involve cell cooperation are not blocked in this way¹⁶. Thus, the effect of adding N-BSA or C-BSA on the response of OFd primed T cells to OFd was ascertained. Figure 2 shows that the addition of $10 \mu\text{g ml}^{-1}$ of N-BSA or C-BSA suppressed the response to $1 \mu\text{g ml}^{-1}$ OFd. Significant but not complete suppression was obtained with $1 \mu\text{g ml}^{-1}$ N-BSA or C-BSA (data not shown). Analogous results were obtained with N or C hapten coupled to poly-D-glutamic acid, or to succinylated BSA (data not shown). As control, the helper cell response to KLH was also assayed with the same pool of helper cells, to exclude the possibility that the suppression of the OFd response in the presence of N or C-BSA was due to nonspecific toxicity.

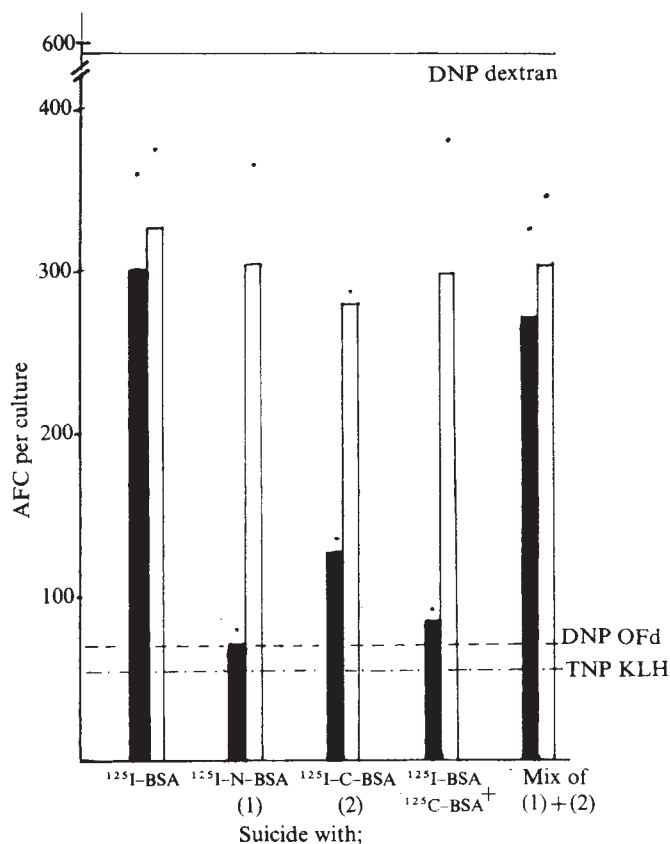


Fig. 3 Antigen induced suicide with radiolabelled N or C-BSA. This was performed in a way similar to that of Byrt and Ada²⁴ and Pearson *et al.*¹³—BSA, N or C-BSA were radiolabelled with ^{125}I using the chloramine-T method, 2–10 μg of each compound was labelled with 1 mCi of carrier-free ^{125}I -iodide (Radiochemical Centre, Amersham). Primed T cells (10^6) (purified by nylon wool) were incubated with $1 \mu\text{g}$ ^{125}I -labelled antigen for 1 h at 0°C in HEPES-buffered Eagle's medium (sometimes containing azide) then washed three times through foetal calf serum, and left overnight at 4°C before culture with OFd and KLH, together with the addition of 5×10^5 anti-T and anti-B serum treated peritoneal macrophages per culture. Dark bar represents response to DNP OFd. Unshaded bar represents the response to TNP KLH. Dot above bar graph indicates the upper limit of one standard error of the mean. Results shown are with 10^5 helper cells from suicided or mixed back helper cell cultures, which were subsequently cultured with normal spleen cells and DNP OFd or TNP KLH. Specific suicide of the response occurred with ^{125}I -N-BSA or ^{125}I -C-BSA. It is interesting that mixing the two suicided populations completely restored the response to OFd. These experiments were performed as a helper cell dose-response titration, using 5×10^5 , 10^5 and 2×10^4 helper cells. For simplicity, the results with 5×10^5 and 2×10^4 are not included, as they corroborated those shown.

implies that a mixture of two cell populations yields a response substantially greater than the sum of the two individual populations. Over the past year, four series of mice have been used for these experiments, each series comprising three groups of mice, which were primed with either the N or the C hapten conjugated to BSA (that is N-BSA), or with OFd. Spleen cells from these mice or mixtures of cells primed to N-BSA or C-BSA were cultured with OFd. Typical results are shown in Table 1 and Fig. 1. Experiments 1 and 2 of Table 1 show that cells primed to N-BSA or C-BSA do not yield significant numbers of helper cells. Mixtures (1:1) of these cells, however, produced responses as good as OFd primed cells and sometimes significantly better (Fig. 1). Since neither N nor C primed cells yield measureable help, it is not possible to calculate the exact degree of synergy, but this can be estimated to be at least 25-fold, and often 500-fold (Table 1). That the interacting N and C primed cells were T cells is shown by experiment 2 of Table 1 and Fig. 1, which indicates that nylon wool filtered spleen cells ($<10\%$ B), or anti-B and complement-treated spleen cells ($<3\%$ B) cooperated even more efficiently with B cells than helper cells obtained from unpurified spleen (experiment 1). Experiments

Table 1 Synergy between N- and C-primed T cells in the generation of helper cells to OFd

Cells cultured	Helper cell induction			Cell cooperation	
	Treatment	Antigen	Helper cells transferred	Antigen	Anti-DNP response (AFC per culture)
(1) OFd primed spleen	nil	OFd	10 ⁵	DNP OFd	173 ± 22
N-primed spleen	nil	OFd	10 ⁵	DNP OFd	13 ± 10
C-primed spleen	nil	OFd	10 ⁵	DNP OFd	0
N-+ C-primed spleen	nil	OFd	10 ⁵	DNP OFd	217 ± 33
(1 : 1) —	—	—	—	DNP OFd	3 ± 43
				DNPPOL	553 ± 160
				nil	27 ± 33
(2) OFd primed spleen	Nylon wool	OFd	5 × 10 ⁵	DNP OFd	320 ± 122
			10 ⁵	DNP OFd	417 ± 65
	Nylon wool		2 × 10 ⁴	DNP OFd	313 ± 100
N-primed spleen	Nylon wool	OFd	5 × 10 ⁵	DNP OFd	187 ± 53
			10 ⁵	DNP OFd	110 ± 47
C-primed spleen	Nylon wool	OFd	5 × 10 ⁵	DNP OFd	173 ± 30
			10 ⁵	DNP OFd	123 ± 62
N-primed + C-primed	Nylon wool	OFd	5 × 10 ⁵	DNP OFd	743 ± 105
(1 : 1) —	—	—	10 ⁵		717 ± 82
			2 × 10 ⁴	DNP OFd	267 ± 17
			—	DNP OFd	127 ± 25
				TNP T ₄	920 ± 110
				nil	113 ± 54

CBA mice bred at University College or at the ICRF Animal Unit at Mill Hill were used at 70–210 d of age. For use as donors of primed spleen cells, they were injected intraperitoneally with 25 µg of OFd, N- or C-BSA emulsified in Freund's complete adjuvant. Usually the N or C hapten was conjugated to succinylated BSA. 15 × 10⁶ spleen cells were cultured with 1 µg ml⁻¹ OFd for 4 d to yield helper cells. Cell recoveries were variable from 10–45%, but averaged 30%. Washed viable (Trypan blue exclusion) cells from these cultures were mixed in the numbers indicated with 15 × 10⁶ unprimed CBA spleen cells and 1 µg ml⁻¹ DNP OFd and cultured for a further period of 4 d before assay for anti-DNP antibody forming cells using the Cunningham technique¹⁴. Preliminary experiments had indicated that the two periods of 4 d and 1 µg ml⁻¹ of antigen were optimal. Eleven experiments of the type shown as experiment 1 have been performed. No helpers were obtained with N or C primed cells at other times of culture (1–6 d), or other concentrations of OFd.

In experiment 2, spleen cells from OFd, N or C primed mice were passed through nylon wool columns²⁰ to purify T cells. Contamination with B cells, as assessed by fluoresceinated rabbit anti-mouse Ig antibody was 5–9% in replicate experiments. Because the nylon wool columns also remove macrophages, essential for helper cell induction²¹ 5 × 10⁵ peritoneal exudate macrophages (obtained 3 d after the injection of starch), purified by treatment with anti-B serum and complement²¹ were added to each helper cell culture. The exact degree of synergy is difficult to estimate, as neither N nor C primed cells yield detectable help. A crude approximation of the degree of help may be obtained by comparing the smallest numbers of (N and C) mixed cells which provide help, for example, 2 × 10⁴, with those unmixed that do not, for example 5 × 10⁵, giving a synergy ratio of greater than or equal to 25. DNPPOL (dinitrophenylated polymeric flagellin) and TNPT₄ (trinitrophenylated phage T₄) are thymus-independent antigens.

The technique of radioactive antigen-induced suicide was also used to verify the requirement for both N-hapten and C-hapten reactive cells to generate helper cells to OFd. Incubation of OFd primed spleen cell or purified T cells with highly radioactive (¹²⁵I-labelled) N or C-BSA suppressed the helper response to OFd. In contrast, the responses of the same cell pool to KLH was unimpaired. ¹²⁵I-BSA was not suppressive. Mixing the N-suicided and the C-suicided populations (each of which should contain an intact population of cells directed against the other hapten) restored the OFd helper response to normal levels, thus providing further evidence for a cooperative interaction between the two hapten reactive cells.

Three independent experimental approaches have thus all indicated that N and C reactive T cells interact to generate helper cells to OFd. It seems then that there is T–T interaction in the development of helper cells, just as in other T cell responses—the graft versus host response⁶ and the T killer response^{7–10}. Other studies also support our evidence for T–T interaction in the generation of helper cells. Either anti-lymphocyte serum (ALS) treatment or adult thymectomy (ATX) markedly reduces the thymus-dependent antibody response to tobacco mosaic virus¹⁷. Furthermore, Hunter, Kappler and colleagues have also shown that both ALS and ATX diminish helper responses to sheep red cells, but that both procedures are needed to abolish the helper response^{18,19}. Our studies *in vitro*, using keyhole limpet haemocyanin as antigen, indicated that defined populations of T₁ and T₂ cells purified from the spleen (by treatment with ALS and by adult thymectomy respectively) do not yield helper cells unless mixed together (unpublished data), in contrast to normal spleen which contains both T₁ and T₂ cells. Thus the interaction of subpopulations of T cells is of importance in the generation of helper cells, just as it is in the development of graft versus host and the cytotoxic response.

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Concurrent inhibition by chlorpromazine of concanavalin A-induced lymphocyte aggregation and mitogenesis

A PLANT lectin from extract of Jack bean (concanavalin A) is known to initiate transformation *in vitro* of T lymphocytes into large, proliferating blast-like cells¹. Knowledge of the mechanism whereby con A induces such changes is incomplete, but a necessary first step seems to involve the binding of con A to receptors on the surface membrane of the lymphocyte. Within minutes of binding of a mitogen, recognisable alterations in lymphocyte surface membrane physiology have been noted. These include increased phospholipid incorporation into the membrane², increased fluxes of ionic potassium³ and calcium⁴, and increased uptake of nucleosides⁵, sugar⁶, and amino acids⁷. In addition, con A has been reported to increase the fluidity of lymphocyte membranes after binding⁸. Internalisation of con A is not required for lymphocyte activation so that con A binding to the lymphocyte surface membrane of T cells seems to be sufficient to induce blastogenesis⁹.

Less attention has been paid to the fact that con A also induces an aggregation or clustering of mononuclear cells following its addition to *in vitro* culture. At optimal mitogenic concentrations, this process is not an immediate agglutination but occurs progressively over several hours in culture. It has been suggested that such cluster formation may be a necessary prerequisite for blast transformation induced by a mitogen, as cells immobilised in single cell suspensions in agar will not transform¹⁰, and increased cell density¹¹, presumably augmenting cell-cell interaction, enhances mitogenesis. In accordance with this hypothesis we have found that similar concentrations of the surface membrane-active drugs, chlorpromazine (CPZ) will inhibit both the blastogenic response as well as the clustering of mouse spleen cells by con A.

The effect of CPZ on con A-induced blastogenesis is shown in Fig. 1. Concentrations of CPZ >0.005 mM totally inhibited the uptake of ³H-thymidine by con A-stimulated cultures. Concentrations of CPZ <0.001 mM had little effect.

When dealing with pharmacological inhibition studies such as this it is imperative that any cytotoxic effects of the drugs themselves be evaluated. Mouse spleen cells were therefore incubated with inhibiting concentrations of CPZ for 24–48 h in the presence or absence of con A. No loss of viability was seen at concentrations ≤ 0.05 mM by Trypan blue exclusion or ⁵¹Cr release assays. In addition, if spleen cells were pre-incubated for 2 h in the presence of concentrations of the drug (0.01 mM) sufficient to inhibit mitogenesis, washed extensively, and then cultured with con A, there was no difference in the blastogenic response compared with untreated control cells (data not shown). Thus, CPZ in these concentrations was not detectably cytotoxic to lymphocytes and its effect was reversed by removal of the drug. To further prove viability and characterise the inhibition of blastogenesis, CPZ was added at intervals after incubation of spleen cells with con A. If CPZ was added with con A or 2 h later, marked depression of thymidine incorporation was observed, whereas if CPZ was added after 24 h a response very similar to cultures lacking CPZ was observed (Fig. 2). This experiment provides additional proof of viability, as ³H-thymidine incorporation in response to con A proceeded unaltered in the presence of the drug. In addition, the inhibitory effect of the drug seems not to derive from an inhibition of DNA synthesis, that is, once the con A-induced intracellular message had been sent and the lymphocyte was committed to respond, the addition of the drug had no effect on the subsequent blastogenic response.

Con A-treated lymphocyte cultures were observed by light microscopy. Aggregation was far advanced by 12 h in the

absence of CPZ. Con A-induced lymphocyte aggregation was inhibited by CPZ at concentrations equal to that which inhibits blastogenesis. CPZ (0.01 mM) added to con A cultures after 12 h, however, did not disperse already formed cell clusters.

One possible mechanism of both inhibition of blastogenesis and of cluster formation could be the inhibition by CPZ of binding of con A to the surface of the lymphocyte. To test this possibility, binding studies using ³H-labelled con A were carried out. A 1 : 5 dilution of ³H-con A (80–92 Ci mM⁻¹) was made and diluted in a solution of 100 μ g ml⁻¹ unlabelled con A. This was added to a population of spleen cells (5×10^6 cells ml⁻¹), incubated for 30 min at 37 °C, washed three times with 1% albumin solution in phosphate-buffered saline, collected as above, and counted in a scintillation counter. The

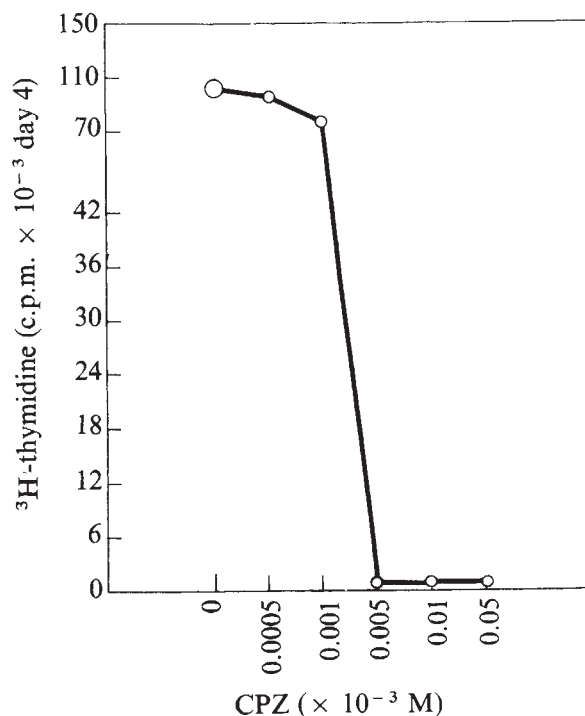


Fig. 1 CPZ dose-response inhibition of con A-induced ³H-thymidine incorporation of murine (C3H/HeJ) spleen cells: 5×10^5 spleen cells were cultured in 0.2 ml medium 199 (GIBCO) containing 7.5% heat-inactivated human pooled serum and 1% penicillin and streptomycin. Cultures were carried out in triplicate in 96-well, flat bottom microtitre plates (Falcon). CPZ (Smith, Kline and French) was added in appropriate concentration immediately before addition of con A (5μ g ml⁻¹). At 18–24 h before collection, each well was pulsed with 1 μ Ci ³H-thymidine (6.7 Ci mM⁻¹). After 96 h culture, cells were collected in a 12-place mechanical collector (Otto Hiller), dried and counted in a Beckman LS330 scintillation counter. Each point represents the mean of triplicate cultures not differing by more than 10%.

concentrations of CPZ found to inhibit blast transformation had no effect on the binding of con A to the lymphocyte cell surface; however, α -methylmannose, an inhibitor of con A binding, did inhibit binding. As binding of ³H-con A is unaffected by CPZ, some event subsequent to lectin binding but necessary for both aggregation and blastogenesis must be at work. This is further supported by considerable data that CPZ acts primarily on the membranes of nerve, muscle and lymphocytes.

Studies of lymphocyte activation have focused on the role of cyclic AMP¹² and cyclic GMP¹³ as well as mitogen-induced

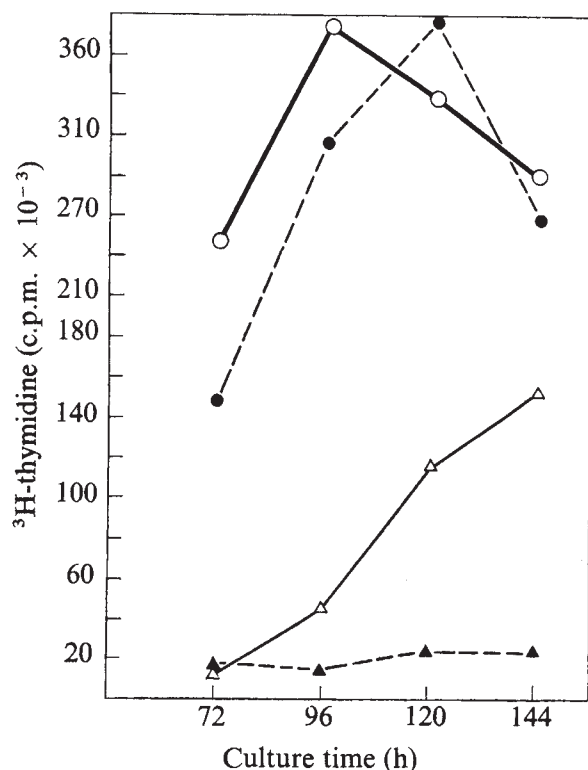


Fig. 2 Effect of timed addition of CPZ (0.01 mM) on con A-induced ^3H -thymidine incorporation. Cells were cultured and pulsed as in Fig. 1. Cultures were collected at times indicated on the abscissa. Con A ($5 \mu\text{g ml}^{-1}$) was added to all cultures (5×10^5 cells) at initiation of culture. CPZ (0.01 mM) was added at various times after culture. ○, No CPZ added; ▲, CPZ added with con A; △, CPZ added 2 h after con A; ●, CPZ added 24 h after con A.

changes in Ca^{2+} flux¹⁴. CPZ is a well established Ca^{2+} -blocking agent producing inhibition of Ca^{2+} fluxes in muscle¹⁵ and adrenal preparations¹⁶. This pharmacological action of CPZ has been shown to be reversed by high Ca^{2+} concentrations in the media¹⁷. If, however, lymphocytes are cultured in media containing 5 or 10 mM calcium no reversal of CPZ inhibition of con A-induced blastogenesis was observed (data not shown).

CPZ has also been shown to inhibit hormonally stimulated increases in adenylate cyclase activity of rabbit heart tissue without affecting the base level of enzyme activity¹⁸, and to decrease the amphetamine-induced increase of cyclic GMP of murine cerebellum¹⁹. Preliminary data indicate that addition of a range of concentrations of cyclic AMP, or its dibutyl derivative (10^{-6} – 10^{-3}M) did not reverse CPZ-induced inhibition of transformation or lymphocyte aggregation (data not shown). Cyclic GMP or its dibromo derivatives in a dose range of 10^{-9} – 10^{-3}M either added to cultures with con A and CPZ or added to cells for 15 min before culture with con A and CPZ, also did not reverse the inhibitory effect of CPZ (data not shown). It seems therefore that a membrane phenomenon not reversed by Ca^{2+} , cyclic AMP or cyclic GMP, is involved in the inhibition of con A-induced lymphocyte transformation by CPZ.

The cellular requirements for mitogen stimulation and their interactions in the activation process are possible targets for CPZ action. Highly purified human T cells show a markedly reduced response to mitogen-induced blastogenesis (J.R.S., and S. Hatfield, unpublished). This response, however, could be restored by the addition of isologous, allogeneic or even xenogeneic macrophages to mitogen cultures. This suggests that the macrophage plays a significant role in the response of T cells to mitogens. Additional support for the importance

of cellular interactions in lymphocyte activation by mitogens is given by Moorhead¹¹ who demonstrated an inverse and linear relationship between the surface area on which equal numbers of lymphocytes were cultured and the log of ^3H -thymidine incorporation into mitogen-stimulated cells. Kondrocki¹⁰ has shown that isolated 'single-cell' suspensions of lymphocytes in agarose respond poorly to mitogen but that addition of erythrocytes or erythrocyte stromata to such preparations markedly increases the blastogenic response. Implicit in the above observations is that cell membrane interactions play an important role in mitogen activation. CPZ, as shown above, inhibits mitogen-induced lymphocyte clustering seen in con A-stimulated cultures and thus removes the opportunity for cell-cell interactions.

Whether aggregation and blastogenesis are a manifestation of a common lectin-induced lymphocyte membrane perturbation or whether each is an independent consequence of mitogen binding is not known. Further studies to answer this question, utilising a surface membrane pharmacological approach to soluble and cellular *in vitro* antigen stimulation, in addition to mitogen activation, are in progress.

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Temperature dependence of phenoxybenzamine effects and the adrenoceptor transformation hypothesis

ADRENOCEPTORS can be classified into two types, α or β , according to their sensitivity to agonists and antagonists. It has been claimed that the β -adrenoceptors mediating the effect of adrenaline on the contractility of frog isolated heart are changed to α -adrenoceptors by lowering the temperature from 24 to 14 °C (ref. 1). The evidence for this suggestion was that a high concentration of the α -adrenoceptor-blocking drug phenoxybenzamine inhibits the effect of adrenaline at the low temperature but not at the higher temperature, and that the frog heart binds almost twice as much phenoxybenzamine at

Table 1 Inotropic potencies of isoprenaline, adrenaline and phenylephrine (ED_{50}), and potency of propranolol (expressed as the dose ratio of isoprenaline) on the frog isolated ventricle at 24 and 14 °C

	Temperature (°C)	No. of expts	Negative log molar ED_{50} (mean $\pm$ s.e.)	log Dose ratio of isoprenaline
Isoprenaline	24	7	8.48 $\pm$ 0.31	—
Isoprenaline	14	9	8.76 $\pm$ 0.50	—
Isoprenaline + 0.34 μ M propranolol	24	8	6.70 $\pm$ 0.11	1.78
Isoprenaline + 0.34 μ M propranolol	14	9	6.97 $\pm$ 0.12	1.79
Isoprenaline + 3.4 μ M propranolol	24	6	5.84 $\pm$ 0.02	2.64
Isoprenaline + 3.4 μ M propranolol	14	6	5.85 $\pm$ 0.02	2.91
Adrenaline	24	9	6.66 $\pm$ 0.40	—
Adrenaline	14	9	7.37 $\pm$ 0.62	—
Phenylephrine	24	4	5.29 $\pm$ 0.23	—
Phenylephrine	14	5	5.46 $\pm$ 0.22	—

Strips of frog ventricle were suspended in a solution consisting of 111 mM NaCl, 2.4 mM $NaHCO_3$, 1.9 mM KCl, 1.1 mM $CaCl_2$, 0.07 mM NaH_2PO_4 , and 10 μ g ml⁻¹ disodium edetate. Contractions were elicited with a Grass stimulator (1–3 ms, voltage just above threshold) at a rate of 0.3 Hz (24 °C) or 0.2 Hz (14 °C) and recorded isometrically. Potencies of the agonists were determined from cumulative dose–response curves. Propranolol was present 60 min before isoprenaline was added. Potency of the antagonistic drug is expressed as the dose ratio: this is the ratio of the potency of the agonist in the presence of the antagonistic drug and in its absence. Frog experiments were carried out between the months of September and February.

the low temperature than at the higher temperature. Phenoxybenzamine, particularly in high concentration, does not, however, specifically block α -adrenoceptors, and the purpose of the present experiments was to test the hypothesis of the receptor transformation by procedures not dependent on the use of this drug.

The adrenoceptor is a descriptive term and its nature is unknown. A response is said to be mediated by α -adrenoceptors when the relative potency of a series of agonists shows that adrenaline is highest, phenylephrine is somewhat lower, and isoprenaline is very low, and when the response is susceptible to blockade by a low concentration of an α -adrenoceptor-blocking drug. A response is said to be mediated by β -adrenoceptors when the relative potency of a series of agonists shows that isoprenaline is highest and phenylephrine lowest, and when the response is susceptible to blockade by a low concentration of a β -adrenoceptor-blocking drug. The present experiments used these criteria to test the validity of the adrenoceptor transformation hypothesis; the results do not support the concept that β -adrenoceptors are changed to α -adrenoceptors at low temperature. Adenylate cyclase coupled β -adrenoceptors in frog heart² and erythrocytes³ did not change with temperature.

Table 1 summarises the results of experiments to determine the inotropic potencies of isoprenaline, adrenaline and phenylephrine at 24 and 14 °C. Lowering the temperature had little effect on the potencies of the sympathomimetic drugs. Table 1

also shows the antagonism of isoprenaline by the β -adrenoceptor-blocking drug propranolol; the blocking effect of this drug was not altered by the change in temperature. It seems from these results that low temperature does not change the β -adrenoceptors mediating the inotropic effect on the frog heart.

Phenoxybenzamine is a highly lipid-soluble compound which is capable of alkylating nucleophilic centres after forming an aziridinium ion on neutralisation⁴. In isolated mammalian heart preparations nanomolar concentrations of phenoxybenzamine block α -adrenoceptors⁵ and micromolar concentrations have additional effects, such as an acetylcholine antagonism at muscarinic sites⁶; an inhibition of tyramine effects⁷, probably caused by blockade of neuronal uptake sites⁸; a potentiation of noradrenaline effects⁷, probably the result of blockade of neuronal and extraneuronal uptake sites⁹; and a reduction in heart rate and inhibition of the chronotropic effect of noradrenaline, which is not the result of adrenoceptor blockade¹⁰.

In agreement with previous observations¹ it was found that 7.4 μ M phenoxybenzamine inhibits the response of the frog heart to adrenaline at 14 °C, that the inhibitory activity on incubation with phenoxybenzamine at low temperature persists when the temperature is raised to 24 °C, and that phenoxybenzamine had no effect when incubation was carried out in the presence of 10 μ g ml⁻¹ of the competitive α -adrenoceptor-

Table 2 Effect of interaction of phenoxybenzamine with acetylcholine, tyramine and noradrenaline on contractility of the frog isolated ventricle and the guinea pig isolated left atrium at 14 °C and 24 or 31 °C

	Treatment	Agonist	Temperature (°C)	log Dose ratio of agonist
Frog	0.1 μ M atropine	Acetylcholine	Treatment + assay, 24	2.81
Frog	0.1 μ M atropine	Acetylcholine	Treatment + assay, 14	2.58
Frog	2.9 μ M phenoxybenzamine	Acetylcholine	Treatment + assay, 24	2.30
Frog	2.9 μ M phenoxybenzamine	Acetylcholine	Treatment, 24; assay, 14	2.16
Frog	2.9 μ M phenoxybenzamine	Acetylcholine	Treatment + assay, 14	0.79
Frog	2.9 μ M phenoxybenzamine	Acetylcholine	Treatment, 14; assay, 24	0.36
Frog	2.9 μ M phenoxybenzamine	Acetylcholine	Treatment + assay, 31	1.96
Guinea pig	0.74 μ M phenoxybenzamine	Acetylcholine	Treatment, 14; assay, 31	0.60
Guinea pig	0.74 μ M phenoxybenzamine	Acetylcholine	Treatment + assay, 31	0.84
Guinea pig	7.4 μ M phenoxybenzamine	Tyramine	Treatment 14; assay, 31	0.20
Guinea pig	7.4 μ M phenoxybenzamine	Tyramine	Treatment + assay, 31	-1.16
Guinea pig	7.4 μ M phenoxybenzamine	Noradrenaline	Treatment, 14; assay, 31	-0.08

Strips of guinea pig left atrial appendage were suspended in a solution consisting of 137 mM NaCl, 11.9 mM $NaHCO_3$, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.36 mM NaH_2PO_4 , 5.55 mM glucose and 10 μ g ml⁻¹ disodium edetate which was aerated with 5% CO_2 in O_2 . Contractions were elicited at a rate of 1 Hz (31 °C) or 0.2 Hz (14 °C). See Table 1 for details. Incubation with phenoxybenzamine was for 40 min after which bath fluid was repeatedly changed before agonists were assayed. A positive dose ratio indicates inhibition, and a negative dose ratio potentiation, of the inotropic effect of the agonist.

blocking drug phentolamine. It was also found, however, that $10 \mu\text{g ml}^{-1}$ of the membrane stabilisers procainamide or quinidine prevented the phenoxybenzamine effect and that $7.4 \mu\text{M}$ phenoxybenzamine inhibited the effect of isoprenaline, although isoprenaline acted on β -adrenoceptors. From these results we conclude that the frog heart at low temperature is sensitive to an inhibition by phenoxybenzamine of Ca^{2+} movements which are the result of β -adrenoceptor stimulation¹¹. Although the effect was persistent, it may not be based on covalent bond formation. Tissues have a high binding capacity for unchanged phenoxybenzamine¹², and lowering of temperature stabilises the intermediate aziridinium ring and greatly reduces its reactivity⁴.

Other experiments measured the negative inotropic effect of acetylcholine and showed that phenoxybenzamine antagonised this effect (Table 2). Unlike atropine, phenoxybenzamine was inactive at low temperature. The behaviour of phenoxybenzamine was persistent: incubation at 24°C led to blockade, and incubation at 14°C did not, whether acetylcholine was assayed at 24 or 14°C .

Studies with guinea pig atria also demonstrated the inactivity of phenoxybenzamine at low temperature. Phenoxybenzamine pretreatment at 31°C , but not at 14°C , inhibited the effect of tyramine and potentiated the effect of noradrenaline, and there was no inhibition of the noradrenaline effect after phenoxybenzamine treatment at 14°C (Table 2).

Our results show that phenoxybenzamine inhibits the effect of adrenaline on the frog heart at low temperature by an action which does not involve α -adrenoceptors and that the increased tissue retention of the drug is not associated with adrenoceptor blockade. Such experiments cannot be interpreted as indicating that β -adrenoceptors are changed to α -adrenoceptors at low temperature. Our experiments to test the relative effects of agonists at high and low temperature clearly indicate that the nature of the adrenoceptors changes little with altered temperature.

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Effects of sodium on solute transport between compartments in intestinal mucosal epithelium

THE uptake of amino acids and of some monosaccharides into the intestinal mucosal cell across the brush-border membranes requires sodium in the intestinal lumen^{1–3}. An hypothesis of the existence in brush-border membranes of systems for transport that depend on Na—for example, the 'Na gradient' hypothesis⁴—although necessary, is not sufficient to account for all the effects of Na on epithelial transport. Whereas in *in vitro* conditions⁵ the accumulation of amino acids and monosaccharides within the epithelium depends markedly on Na in the intestinal lumen, movement of these substances from the lumen into the blood in *in vivo* conditions is less dependent on Na in the lumen^{6–9}.

Using a vascularly-perfused preparation of frog intestine, we have shown that removal of Na from the vascular bed causes an inhibition of solute transfer into the blood that is additional to that resulting from Na-free conditions in the lumen^{8,10}; here, we present further evidence to distinguish between the effects of Na on epithelial, as distinct from membrane (that is, brush-border) transport processes.

Removal of Na from the vascular perfusate decreases the extracellular space as measured by the washout into the circulation of labelled sucrose initially added to the vascular perfusate (Fig. 1). Addition of Na to the vascular perfusate increases the rate of efflux of intracellular 3-O-methyl-D-glucose (3-O-MeG) into the vascular bed, the cells initially having been loaded from the vascular side in the presence of an extracellular marker (Fig. 2a).

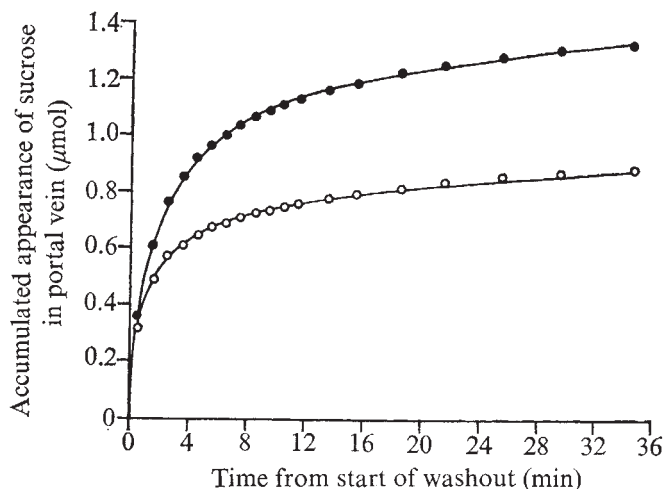


Fig. 1 Effect of presence (●) or absence (○) of sodium in the arterial infusate on the washout into the portal vein of the extracellular marker sucrose. U-¹⁴C labelled sucrose (1 mM; Radiochemical Centre, Amersham) was infused into the mesenteric artery for 30 min. A non-radioactive perfusion medium was abruptly switched into the arterial bed and the washout collected in 1-min fractions. Accumulated appearance of sucrose during washout is plotted against time. Choline substituted for sodium for the period of Na-free vascular perfusion; the lumen was perfused with Na-free Ringer's solution containing 5×10^{-5} M phlorizin. Control and experimental periods were carried out on the same vascularly-perfused small intestine of *R. ridibundus*¹⁰. Note the substantial increase in extracellular space when Na is present in the vascular perfusate: other experiments showed that neither Li nor K could substitute for Na. Vascular flow rate was 2.73 ml per min per g dry weight; that in the lumen was 0.83.

After Na-free perfusion of lumen and vascular bed, return of Na to the lumen increased the rate of efflux of 3-O-MeG from the cells into the vascular bed (Fig. 2b); although the effect of Na in the lumen was not as great as that seen with return of Na to the vascular bed, the observation that Na in the lumen may partially substitute for Na in the vascular bed strongly suggests that the effects of Na depend on events within the epithelium and not in the underlying muscle. We also found that the total quantity of 'intracellular' 3-O-MeG loaded into the cells from the circulation is increased by 43% on addition of Na to the vascular perfusate.

To investigate the effects of Na in the vascular perfusate on the translocation of intracellular amino acids into the blood, a high concentration (10 mM) of the relevant amino acids in the lumen was used to saturate the brush-border amino acid uptake systems¹⁴; any additional appearance of amino acid in the perfusate after subsequent addition of peptide to the lumen will then be a consequence of separate peptide uptake. We found that the removal of Na from the vascular perfusate results in a large though reversible inhibition (67%) of the transfer of amino

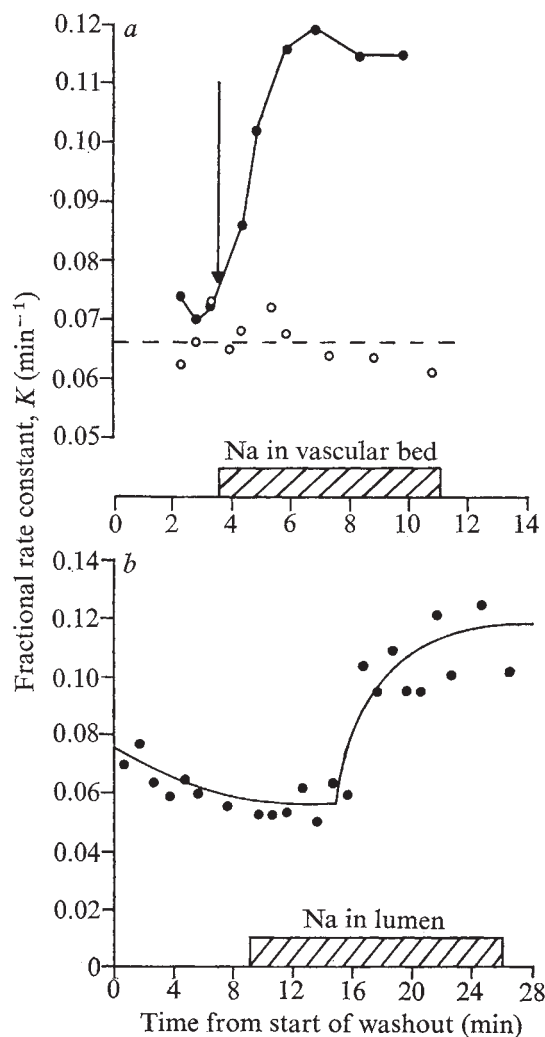


Fig. 2 *a*, Effect of introducing sodium into the vascular bed (arrow) on fractional rate constant for 'intracellular' 3-O-MeG unloading from the small intestine into the effluent collected from the portal vein. Rate of 'intracellular' 3-O-MeG appearance in the circulation was calculated from the difference in the fractional appearance during washout (see refs 11 and 12) of $1\text{-}^3\text{H}$ -3-O-MeG and of an extracellular marker, $\text{U-}^{14}\text{C}$ -sucrose (both from Radiochemical Centre, Amersham) following constant infusion of both substrates at a concentration of 1 mM into the mesenteric artery for 30 min. Scintillation counting of aliquots of 1-min fractions of the effluent appearing in the portal vein was as described previously¹⁰. Fractional rate constants (K) were calculated according to Caldwell and Keynes¹³. Control Na-free ($\circ$) and experimental periods (Na where indicated, $\bullet$) were carried out on the same vascularly-perfused small intestine of *R. ridibunda*¹⁰, thus demonstrating reversibility following Na removal. Ringer's solution was as given in ref. 10 except that Li rather than K substituted for Na; however, similar findings were made when Na was replaced with choline or K. The lumen was perfused with Na-free Ringer which additionally contained 5×10^{-5} M phlorizin to ensure that any observed Na-dependent effect on 3-O-MeG movement could not have arisen at the epithelial brush-border. Vascular flow rate: 2.73 ml per min per g dry weight; luminal flow rate: 0.83. *b*, effect of introduction of Na into the lumen on fractional rate constant (K) for 'intracellular' 3-O-MeG unloading into the effluent appearing in the portal vein. Note that the tissue was loaded from the vascular bed and the experimental protocol was as for *a* except that K rather than Li was substituted for Na. Vascular perfusate was Na-free and that in the lumen contained 5×10^{-5} M phlorizin. Note delay in increase of rate constant when Na is present in lumen. This effect was consistently observed in three preparations. *R. ridibunda*, vascular flow rate: 4.56 ml per min per g dry weight; luminal flow rate: 4.90.

acids initially taken up in the form of peptide into the vascular bed. This is in marked contrast to the uptake of peptide from intestinal lumen which is independent¹⁴ of Na in the lumen.

A further effect of Na is found on solute flux across the epithelium. Unpublished experiments (C.A.R.B.) show that the rates at which both sucrose and 2-deoxy-D-glucose (2-DOG) enter the lumen from the vascular perfusate are substantially increased (by 100% and 97%, respectively) on addition of Na to the lumen. This entry is not influenced by the presence of phlorizin in the lumen. Sucrose is confined to the extracellular space¹⁵ and is suitable as a paracellular probe only because of the extremely low intestinal sucrase activity in the frog⁸. The route taken from the vascular bed to the lumen by 2-DOG is less certain; but whatever it may be, it clearly depends on Na, although it is insensitive to phlorizin, a known competitive inhibitor of monosaccharide transport in brush-border membranes.

The rate of appearance of 3-O-MeG and of 2-DOG in the lumen from constant vascular infusion is presented in Fig. 3. 2-DOG, lacking the hydroxyl group on C-2, is known¹⁶ to have no affinity for the brush-border, phlorizin-sensitive, glucose uptake system, whereas 3-O-MeG behaves like glucose at the brush-border but has the advantage of not being metabolised by frog intestine¹⁷. It is clear that 2-DOG appears at a faster rate in the effluent from the lumen than 3-O-MeG—which initially cannot be detected—and at a time when appreciable amounts of 2-DOG are already present. A pulse of 10 mM D-glucose in the lumen results in a sudden increase in the appearance of both sugars but the effect is proportionately five times greater for 3-O-MeG. The subsequent addition of phlorizin to the lumen increases the rate of 3-O-MeG appearance in the lumen, whereas it has no effect on the movement of 2-DOG.

A simple explanation for these effects is that a significant quantity of the 3-O-MeG that enters the lumen is recaptured by

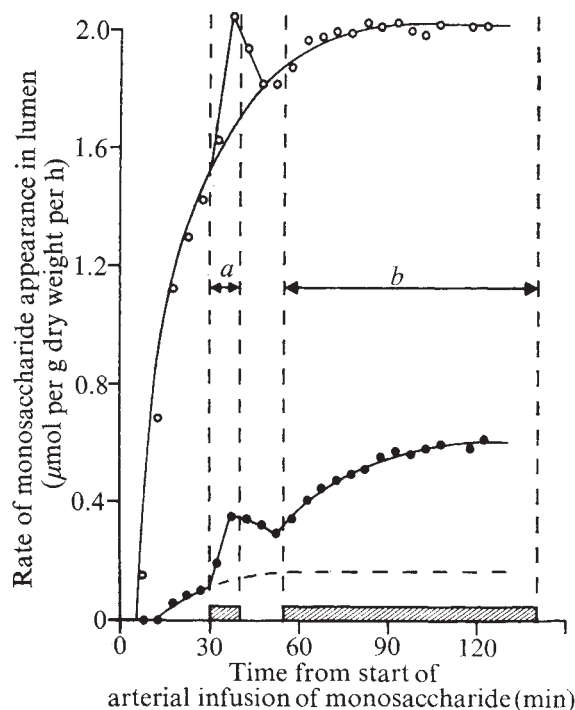


Fig. 3 Rates of appearance in the lumen of $\text{U-}^{14}\text{C}$ -3-O-MeG ($\bullet$) and of $1\text{-}^3\text{H}$ -2-DOG ($\circ$); (Radiochemical Centre, Amersham) after arterial infusion of both sugars at a concentration of 1 mM into the vascularly-perfused small intestine of *R. ridibunda*¹⁰. For the periods indicated, 10 mM D-glucose (*a*) or 5×10^{-5} M phlorizin (*b*) was added to the luminal perfusate. Vascular flow rate: 6.05 ml per min per g dry weight; luminal flow rate: 6.57.

transport systems in the brush-border. Addition to the lumen of phlorizin blocks this reuptake, thus increasing net appearance of the sugar in the lumen. We also find that glucose added to the lumen competes with 3-O-MeG for reuptake, again increasing the net appearance in the lumen of the 3-O-MeG. The absence of an effect of phlorizin and the small effect of glucose on the rate of appearance of 2-DOG in the lumen is also in accord with this explanation, although the movement of 2-DOG into the lumen involves other factors (C.A.R.B., unpublished).

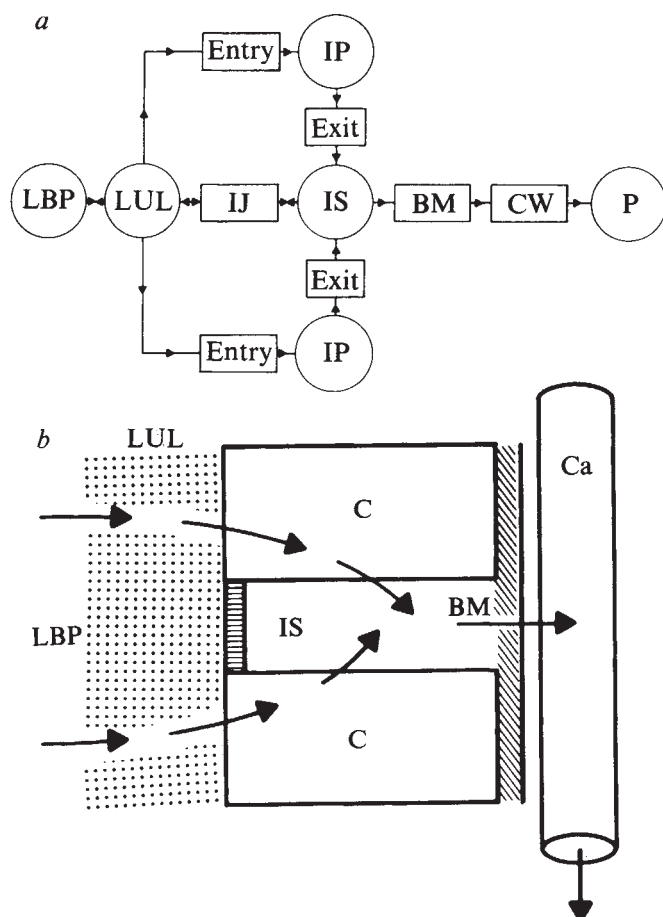


Fig. 4 *a*, Model of relationship between compartments of intestinal epithelium involved in solute transport. Rectangular box, pathway; circle, pool. Two epithelial cells and a single paracellular shunt are depicted. We suggest that Na, in addition to affecting the entry system, increases the intercellular space. LBP, lumen bulk phase; LUL, lumen unstirred layer; IJ, intercellular junction; IS, intercellular space; IP, intracellular pool; BM, basement membrane; CW, capillary wall; P, plasma. *b*, Possible physical interpretation of the model proposed in *a*. C, Epithelial cell; Ca, capillary. Note importance of intercellular space in determining ease of access of substrate to capillary.

The presence of a compartment in the intestinal lumen adjacent to the epithelial sheet, which is unstirred¹⁸⁻²⁰ and from which specific reuptake may occur will minimise escape of substrates from the epithelium into the bulk phase of the lumen contents. Reuptake from such a compartment will be blocked by the presence in the lumen of an inhibitor of brush-border membrane transport, such as phlorizin in the case of monosaccharides, or by the presence of high concentrations of competing substrate. Axon and Creamer²¹ have suggested different interpretations of similar observations.

In Fig. 4 we propose a model that may account for the effects of Na on epithelial transport. It is suggested that, in addition to the known effects on membrane transport at the brush-border (Na gradient hypothesis), Na influences the transfer of substances between compartments in the epithelium. Two of these compartments are extracellular, one intercellular within the epithelium, and the other, a stationary, unstirred layer in the lumen and adjacent to the brush-border membranes. In the light of this model, Csaky's proposal¹⁷ that "a critical intracellular Na concentration is essential" for intestinal (sugar) transport is no longer in direct conflict with the Na gradient hypothesis. The model accounts for the fact that the presence of Na in the vascular bed is necessary for maximal rates of epithelial transport (see also ref. 22). The notion that one effect of Na on epithelial transport is to increase the size of extracellular spaces is in accord with our data on the sucrose space in the epithelium. Such an effect of Na on epithelial transport cannot be seen when the epithelium is disrupted, and has not been found^{23,24}.

The failure to demonstrate effects on solute transfer when the serosal Na is replaced in sheets of intestine *in vitro*⁵ may depend on the presence of a substantial diffusion barrier restricting access of the bathing solution to the epithelium (intramural unstirred layer). This barrier is naturally circumvented by vascular perfusion.

We conclude that in addition to its role in membrane transport, an important function of Na in epithelial transport of solutes is in expanding extracellular spaces within the intestinal mucosa with consequential effects on the clearance of transported substrates from these spaces. The clearance may be forward into the blood stream or backwards into the unstirred layer in the lumen. Our data also suggest that the requirement for Na for this purpose can be completely fulfilled by its presence in the vascular bed, but only partially fulfilled by the addition of Na to the lumen when Na is absent from the vascular perfusate.

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Synthesis and conformations of hypothalamic hormone releasing factors: two QRF-analogues containing backbone N-methyl groups

SINCE its isolation and characterisation^{1,2}, the hypothalamic hormone-releasing factor TRF (pGlu-His-Pro-NH₂) has been the subject of several conformational studies. Most investigations have indicated that the steric characteristics of the three adjacent rings strongly limit the backbone conformational flexibility and support a preference for an extended structure. The importance of each functional group in the molecule in terms of biological potency has been systematically investigated (for review, see ref. 3). With one exception (methylation of the histidyl side chain in the N^τ position) all the chemical modifications which have been made to date were accompanied by a substantial loss of biological activity. We have shown⁴ that most of these modifications do not significantly affect the preferred backbone conformation of the hormone, emphasising the critical role of each functional group in TRF for optimal binding to the receptor sites.

To investigate the 'active' conformation of the hormone we tried to influence the conformational equilibrium of the tripeptide (without affecting its functional characteristics) by preparing an analogue which contains a methylated peptide group between the pyroglutamyl and histidyl residues, [N^αMeHis²]TRF, and a second analogue in which the prolyl residue was substituted by N^αmethylalanyl, [N^αMeAla³]TRF. This latter compound was previously suggested by Burgess *et al.*⁵. From model manipulations it was expected that the first modification would cause a further stabilisation of the molecule in an extended form. Steric interactions between the methyl group and the carbonyl group of the histidyl-prolyl peptide residue, on the one hand, and the β-methylene protons of the histidyl side chain on the other, are expected to affect

slightly the values for the backbone rotational angles of the extended form (ϕ_2, ψ_2) as well as the rotational isomerism of the histidyl side chain (χ_1, χ_2). This modification also prevents the formation of an intramolecular hydrogen bond between the pyroglutamyl-histidyl peptide proton and the N^τ-nitrogen of the imidazole ring, and therefore provides an adequate test of the hairpin turn model⁶ proposed⁷ as the active three-dimensional structure of the hormone. In the second modification, replacement of the prolyl residue by N^αmethylalanyl introduces an additional degree of freedom for the rotational angle ϕ_3 . For a typical ϕ_3 value, however, the N^αmethylalanyl residue sterically simulates the prolyl residue reasonably well.

Both analogues were synthesised by the solid-phase technique using a benzhydrylamine resin of 0.15 mEq g⁻¹ substitution as support. The amino acid residues were introduced as Boc-derivatives (with the exception of ZpGluOH) and coupled in threefold excess in methylenechloride, using dicyclohexylcarbodiimide as coupling agent. The tripeptides were cleaved from the resin by the action of double-distilled hydrofluoric acid at 0 °C over a 45-min period.

The amino acid derivative N^αmethylhistidine was prepared as described previously⁸. Histidine hydrochloride was treated with a threefold excess of benzaldehyde in the presence of N,N-dimethylbenzylamine as base, to give N^αbenzylhistidine which was then methylated by refluxing a formic acid solution of the compound in the presence of an equimolar amount of formaldehyde for 15 min. After cooling, the solution was hydrogenated directly using 10% palladium-charcoal as catalyst. The crude product isolated as the monohydrochloride, was recrystallised twice from water-dimethylsulphoxide and once from water-ethanol (melting point 265–267 °C) and its purity confirmed by thin layer chromatography. It was characterised by mass spectroscopy (peak matching). The N^αMe isomer can easily be differentiated from the N^τ isomer and from the bridged spinacin side product by ion-exchange chromatography as monitored on an amino acid analyser and by mass spectroscopy. The N^αmethylhistidine was then treated with an excess of *tert*-butoxycarbonyl fluoride in a dioxane-water mixture at 0 °C and pH 8.5 until all the starting

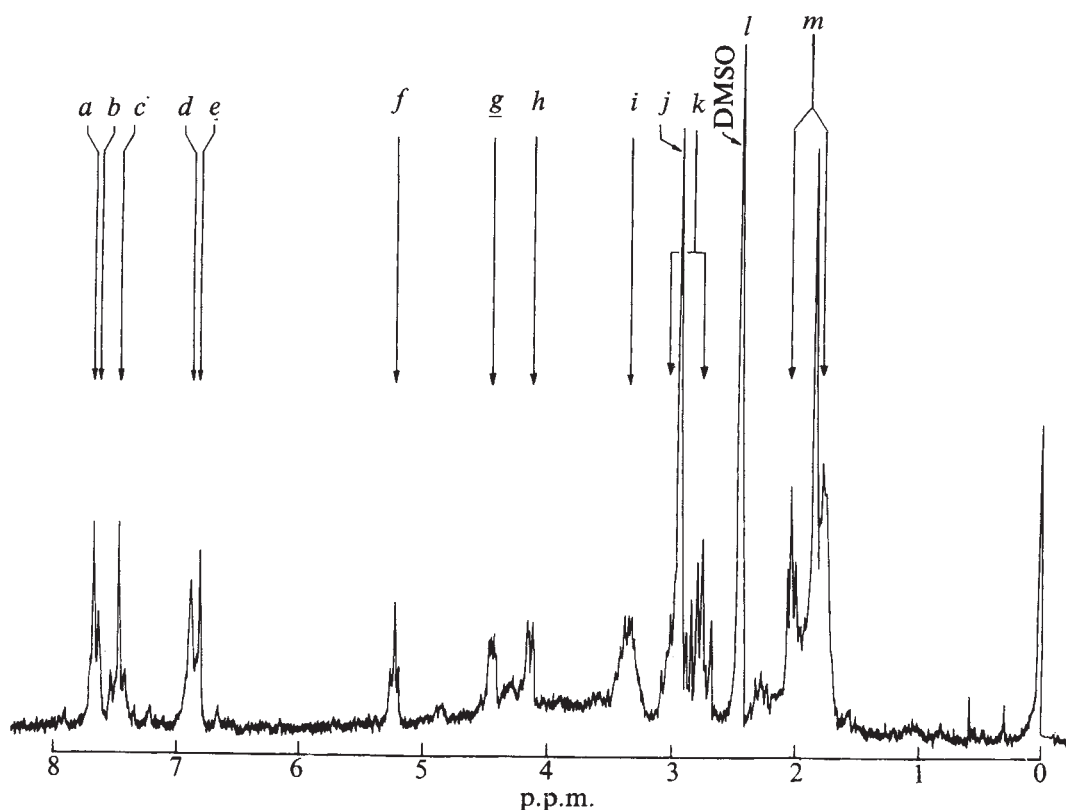


Fig. 1 NMR spectrum (220 MHz) of [N^αMe-His²]TRF acetate in DMSO-d₆ at 22 °C. *a*, pGlu-NH; *b*, ProCONH₂ anti-*trans*; *c*, Im-C²H; *d*, ProCONH₂ *syn-trans*; *e*, Im-C⁴H; *f*, His-C²H; *g*, Pro-C²H; *h*, pGlu-C²H; *i*, Pro-C⁶H₂; *j*, His-N^αMe; *k*, His-C⁶H₂; *l*, DMSO; *m*, pGlu-C⁶H₂, C⁷H₂; Pro-C⁶H₂, C⁷H₂.

material was transformed into N^aMe , N^aBoc , $N^{im}BocHisO^-Na^+$. After concentration of the solution *in vacuo*, it was acidified to pH 3.5 and extracted with a mixture of 20% ethyl acetate in ether, from which the *bis*Boc derivative was obtained as a colourless oil.

After cleavage from the resin, the tripeptides were purified by chromatography on carboxymethyl cellulose eluting with a 0.04 M solution of ammonium acetate, and then by partition chromatography on Sephadex G25 in a butanol-acetic acid-water (4:1:5) system. The products were found to be pure by thin layer chromatography and were characterised by amino acid analysis, mass spectroscopy (peak matching) and nuclear magnetic resonance (NMR) spectroscopy.

The biological potencies of the two analogues were determined as described previously^{9,10}. The *in vivo* bioassay is based on the ability of TRF or active homologues to release thyroid-stimulating hormone (TSH) from the anterior pituitary, thereby increasing the blood level of ¹²⁵I-labelled thyroid hormones in appropriately prepared mice. The potencies relative to pGlu-His-Pro-NH₂ are as follows: [N^aMeAla^3]TRF = $4.5 \pm 1.3\%$; [N^aMeHis^2]TRF = $115 \pm 8\%$. [N^aMeHis^2]TRF was assayed *in vivo* three times giving an average potency of 115% which is not statistically different from that of TRF; this analogue was also found to be indistinguishable from TRF in its ability to stimulate *in vitro* TSH secretion by cultured rat pituitary cells.

The 220-MHz NMR spectrum of [N^aMeHis^2]TRF in DMSO-d₆ is shown in Fig. 1. The following differences from the spectrum of TRF were observed: an upfield shift of the anti-*trans* carboxamide resonance of 0.4 p.p.m.; a quasi-disappearance of the non-equivalence of the Pro-C^β H₂ resonances; and an upfield shift of about 0.1 p.p.m. of the Im-C^αH resonance. These differences strongly suggest that the imidazole-carboxamide interaction which was found in TRF (in DMSO)⁴ is destabilised in the case of [N^aMeHis^2]TRF. This is further supported by the fact that the vicinal coupling constants in the C^α-C^β fragment of the histidyl side chain, which were non-equivalent in TRF ($J_{AX} = 5.3$ Hz; $J_{BX} = 7.6$ Hz), are equivalent in [N^aMeHis^2]TRF ($J_{AX} = J_{BX} = 7.0$ Hz). In terms of the relative populations of the side-chain rotamers, this means that the two *gauche* conformers which were unequally populated in TRF ($b = 0.42$, $c = 0.22$), are equally populated in [N^aMeHis^2]TRF ($b = 0.37$, $c = 0.37$).

A quantitative interpretation of the ABX system of the histidyl side chain in [N^aMeAla^3]TRF was not possible because of overlapping with the methyl resonance. The resonance positions of the anti-*trans* carboxamide proton and of the imidazole C4-proton in this analogue are the same as in TRF, however, which suggests that the imidazole-carboxamide interaction is also favoured in [N^aMeAla^3]TRF dissolved in DMSO.

The differences between the NMR spectra of [N^aMeHis^2]TRF and TRF itself can be explained by a change in the preferred conformation of the histidyl side chain and a small distortion of the preferred backbone conformation. The high biological potency of this analogue in *in vivo* as well as in *in vitro* tests clearly shows that the steric constraints which were introduced on methylation of the pyroglutamyl-histidyl peptide bond did not affect the binding of the hormone to its receptor sites. For the same reason, the hypothetical hydrogen bond between the pyroglutamyl-histidyl peptide proton and the N^π of the imidazole ring does not seem to be a conformational requirement for hormone-receptor interaction.

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Transformation of human cells in culture by N-methyl-N'-nitro-N-nitrosoguanidine

THE development of *in vitro* cell culture systems in which healthy cells can be transformed (converted to malignant cells) by chemicals, and detailed studies of the interaction of chemical carcinogens (or their activated forms) and cells, have aided the study of chemical carcinogenesis. *In vitro* chemical transformation of various rodent cells has been well established¹⁻³. Many attempts have been made to transform various cultured normal or genetically abnormal human cells with chemical carcinogens without success^{4,5}. We have tried to transform human cells from both normal and abnormal individuals and as has been the experience of others, have failed to observe any changes in such cultures. Therefore, the possibility of using continuous lines of human sarcoma cells for chemical transformation was investigated, since certain human sarcoma cell lines are susceptible to transformation by both DNA and RNA tumour viruses⁶⁻⁸. This communication reports results of experiments showing that human osteosarcoma clonal cells can be transformed *in vitro* by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a known potent carcinogen^{9,10} and that these transformed cells produced tumours when injected into NIH nude athymic mice.

A human osteosarcoma clonal cell line designated as TE-85 clone F-5 originally established by McAllister *et al.*¹¹, was obtained from Naval Biomedical Research Laboratory, Oakland, California. The line had cytological and karyological characteristics similar to those of the parent tumour: it grew to high saturation density, formed colonies in agar medium and was aneuploid but did not produce tumours in anti-thymocyte serum-treated hamsters or mice. This line was also found to be highly susceptible to transformation by a feline sarcoma virus⁷

Table 1 Morphological alterations of human cells* treated with MNNG

Passage	Morphological alterations			Growth medium
	MNNG (0.1 µg ml ⁻¹)	MNNG (0.01 µg ml ⁻¹)	DMSO (0.5%)	
0	MNNG treated for 7 d			
1	14	—	—	—
4	35	—	—	—
6	49	±	—	—
7	55	+	—	—
9	72	+	—	—

*The human osteosarcoma clonal line (TE-85, clone F-5) at the 31st subculture.

Table 2 The karyology of human osteogenic sarcoma cells (TE-85 clone F-5) and chemically-treated TE-85 clone F-5 cells

Treatment	Morphology	Range of no. of chromosomes	Polyploidy (%)	Giemsa conventional 'markers'
		Modal no.		
Control (P-13)*	Epithelial-like (polygonal) no foci	46-53 51	38	2-3 Exceptionally long subtelocentrics and 1-2 minutes
MNNG 0.01 $\mu\text{g ml}^{-1}$ (P-13)	Distinct foci of 'piled-up' (+++), refractile cells	72-80 73	99	2-3 Exceptionally long subtelocentrics and 1-2 minutes
MNNG 0.1 $\mu\text{g ml}^{-1}$ (P-13)	Distinct foci of 'piled-up' (+), refractile cells	43-49 49	5	2-3 Exceptionally long subtelocentrics and 1-2 minutes

*Number of subcultures after chemical or DMSO treatment.

and the Kirsten sarcoma virus (KiSV)^{8,12}. Growth and maintenance medium consisted of Eagle's minimal essential medium (MEM) with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 units of penicillin and 100 μg of streptomycin ml^{-1} (MEM+10% FBS).

One day after plating 2×10^5 cells ml^{-1} from the 31st subculture in Falcon plastic Petri dishes, the medium (MEM+10% FBS) was removed and replaced with media containing MNNG at various concentrations ($5.0 \mu\text{g ml}^{-1}$ to $0.01 \mu\text{g ml}^{-1}$) in 0.5% dimethyl sulphoxide (DMSO). The control medium contained 0.5% DMSO. After 7 d treatment with the carcinogen, the cultures were washed, fed again with carcinogen-free growth medium and subsequently passaged by trypsin treatment every 7 d. When changes in morphology and growth patterns appeared, some cultures were fixed in alcohol and stained with Giemsa for further microscopy. Both transformed and untransformed cultures were established as continuous cell lines.

In the MNNG-treated cultures, morphological alterations of cells and abnormal pattern of growth were noted in the sixth subculture, 55-59 d after treatment (Table 1); a similar change was not observed in the control cells treated with DMSO or in the untreated human cells. Doses of $1.0 \mu\text{g MNNG ml}^{-1}$ or greater were lethal. The change was first observed in the cells treated with $0.1 \mu\text{g MNNG ml}^{-1}$; however, the alteration observed in the MNNG ($0.01 \mu\text{g ml}^{-1}$) treated cells became more pronounced after further subcultivation.

The morphological changes observed in these cultures were similar to those observed in a previous study in which KiSV alone transformed TE-85 clone F-5 cells^{8,12}. Foci of transformed cells consisted largely of criss-crossed, randomly oriented, spindle-shaped cells with nuclear and cytoplasmic overlapping which stained heavily with Giemsa (Fig. 1a and b). In contrast, the cellular morphology remained unchanged in the untreated, control human cell line, which continued to grow in monolayers of normal-appearing, fibroepithelial-like cells (Fig. 1c) for 18 subcultures during the 112 d after treatment. An increased growth rate (double that of controls) was observed in the MNNG-treated cells, and they had to be subcultured much more frequently than untreated cells.

The relationship between the transformed and control cells was established by chromosome analysis (Table 2). Untreated cells had remarkable marker chromosomes by conventional staining; the same markers were noted in cells of the transformed cultures. Trypsin-Giemsa banding of cells revealed banded markers previously reported in the parental untreated cells, TE-85 clone F-5 (ref. 13). The fluctuation in polyploidy does not seem to be related to the transformation state, since cells near diploid as well as near triploid were involved.

The MNNG-treated cells, as well as untreated control cells, were tested for their ability to transplant and produce tumours in nude athymic mice. Cells were trypsinised, centrifuged, suspended in growth medium, and inoculated subcutaneously (Table 3). All the inoculated nude mice given 5×10^6 cells from MNNG ($0.01 \mu\text{g ml}^{-1}$) treated cells developed tumours within 2 weeks at the site of inoculation. The tumours were poorly-differentiated sarcomas. A few of the cells were producing a matrix which had some resemblance to osteoid. Moreover, three of five mice given cells from MNNG ($0.1 \mu\text{g ml}^{-1}$) treated cells produced small, persistent nodules. The nodule tumours resembled osteosarcoma and there was considerable matrix resembling osteoid around many of the cells. No tumours developed over a period of 50 d in a group of nude mice inoculated subcutaneously with the same number of cells from the untreated cultures (Table 3). The tumours were progressive and transplantable. Cells established from the tumours resembled the MNNG-treated cells (Fig. 1d).

The human osteosarcoma clonal cells treated with MNNG had the following properties: (1) The MNNG-treated cells were morphologically altered and grew as randomly oriented multilayers; (2) the treated cells showed increased growth rates; (3) when inoculated into nude athymic mice, one concentration ($0.01 \mu\text{g ml}^{-1}$) produced subcutaneous tumours and another ($0.1 \mu\text{g ml}^{-1}$) produced small persistent tumour nodules. It thus seems clear that we have demonstrated the transformation *in vitro* by MNNG of human cells from a continuous cell line. Other continuous cell lines of hamster origin, BHK-21 (ref. 14), of mouse origin, 3T3 (ref. 15) and BALB/c 3T3 (ref. 16), are all susceptible to transformation by

Table 3 Tumours produced in NIH nude mice by human osteogenic sarcoma cells (TE-85, clone F-5) obtained by treatment with MNNG*

Treatment	No. of subcultures after MNNG or DMSO treatment	No. of tumours	Remarks	Pathology
		No. inoculated		
MNNG ($0.01 \mu\text{g ml}^{-1}$)	18	†5/5 (12)‡	Progressive, transplantable tumours	Poorly differentiated sarcomas
MNNG ($0.1 \mu\text{g ml}^{-1}$)	18	†3/5 (12)	Small, persisting nodules	Osteosarcomas
Control	18	0/5		

* 5×10^6 cells inoculated per mouse.

†Tumours were sectioned and diagnosed by Dr D. N. Taylor. Tumours also re-established in tissue cultures.

‡Day tumour first noted.

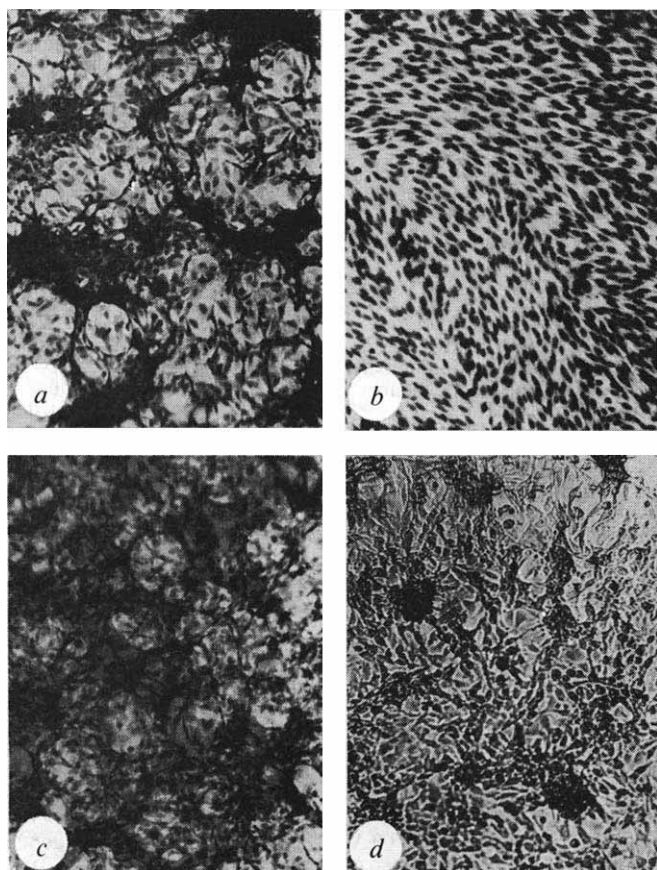


Fig. 1 Human osteosarcoma clonal (TE-85, Clone F-5) cells treated with MNNG for 7 d followed by seven subcultures in nutrient medium. Giemsa stain $\times 39$. Note the morphological alterations in human cells treated with $0.01 \mu\text{g ml}^{-1}$ and $0.1 \mu\text{g ml}^{-1}$ of MNNG: criss-crossed, spindle-shaped cells with nuclear and cytoplasmic overlapping (a and b). a, Human cells treated with $0.01 \mu\text{g ml}^{-1}$ MNNG; b, human cells treated with $0.1 \mu\text{g ml}^{-1}$ MNNG; c, human cells (0.5% DMSO); d, typical field of culture originating from a primary tumour induced by the MNNG ($0.01 \mu\text{g ml}^{-1}$) treated human cells.

both DNA and RNA viruses⁶ as well as by chemical carcinogens⁹. We confirmed the *in vitro* human sarcoma cell transformation by MNNG and also found that a carcinogenic polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)anthracene induced transformation in the human osteosarcoma (TE-85, clone F-5) cell system described here (our unpublished work). Although unfortunately an aneuploid sarcomatous cell line had to be used for this investigation, in view of the fact that little, if any, successful *in vitro* transformation of normal human cells by chemical carcinogens^{4,5} had been reported, the system presented in this report may offer an initial advancement in this direction.

It is interesting to note that the chemically altered human cells like the KiSV-transformed TE-85 cells¹² produced tumours when implanted subcutaneously into NIH nude athymic mice whereas control, untransformed cells did not. Although human TE-85 clone F-5 cells which we used in our experiment had cytological characteristics similar to those of the parent tumour¹¹, they did not form subcutaneous tumours in anti-thymocyte serum (ATS) treated hamsters or ATS-treated mice and did not induce fibrinolytic activity. Subcutaneous inoculation of the original lines TE-85 clone F-5, both from the NBRL repository and our control culture never produced nodules or tumours following implantation of 5×10^6 viable cells. In addition, it is interesting that KiSV-transformed human TE-85 clone F-5 cells induce fibrinolytic activity and yield tumours in ATS-treated hamsters (R. M. McAllister,

personal communication). Studies are in progress to determine whether the chemically altered cells do induce tumours in ATS-treated hamsters and also whether they induce fibrinolytic activity like KiSV-transformed cells.

MNNG, a known potent carcinogen and mutagen that does not require metabolic activation, has been repeatedly demonstrated to transform mammalian cells^{9,10}. Henderson *et al.*¹⁷ have reported an increased transformation frequency of human peripheral lymphocytes following treatment with MNNG, possibly by activation of Epstein-Barr virus. Attempts to activate type C virus or virus expression in MNNG-altered cells are in progress.

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Detection of DNA complementary to pathogenic viroid RNA in exocortis disease

A low molecular weight (about 10^5 daltons) free RNA has been identified as the pathogenic agent of the exocortis disease of citrus (CEV) by Semancik and Weathers¹. This minimal 'infectious' RNA (viroid), characterised by a highly ordered structure rich in G-C base pairs² does not contain polyadenylate sequences³ nor can it be translated *in vitro*⁴. The CEV-RNA induces the identical symptom expression in tomato and potato as the spindle tuber viroid⁵, thus supporting the class relationship of these unique pathogenic RNAs. The limited genetic potential of these extremely low molecular weight pathogenic RNAs lends credence to the suggestion of host-dependent synthesis⁶ and the homology of the pathogenic CEV-RNA with the host genome. This relationship is supported by the detection of a CEV-like DNA, reported here, presumably resulting from the action of host polymerase⁷. The final pathogenic response induced by the viroid RNA might then reflect simply an aberrant expression of the host genome. These functions, resulting in pathogenic RNA synthesis by means of a DNA intermediate as well as the intimate interaction with

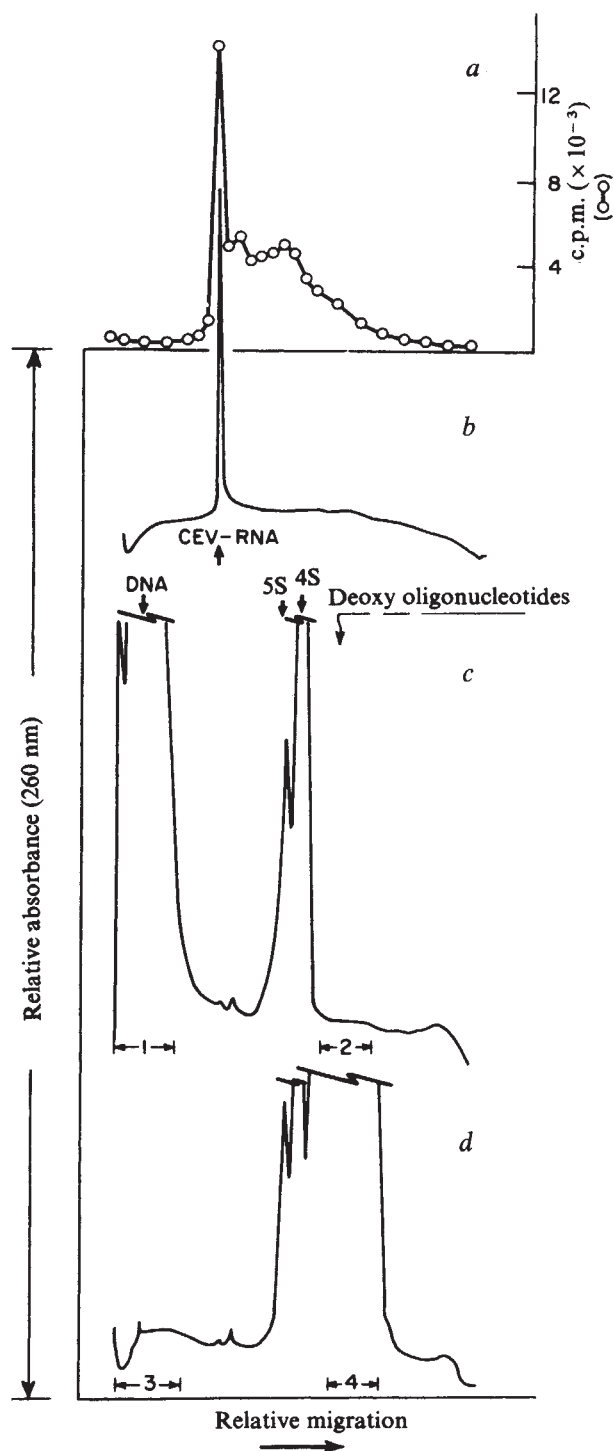


Fig. 1 Electrophoresis on 5% polyacrylamide gels of ^{125}I -labelled CEV-RNA (a) as iodinated from a purified preparation as shown in the $A_{260\text{nm}}$ scanning pattern (b) and a DNA-rich preparation from exocortis-infected *Gynura aurantiaca* before (c) and after (d) treatment with pancreatic DNase I. CEV-RNA was purified as previously reported² and iodinated with ^{125}I as reported⁹ to a specific activity of about 10^7 d.p.m. μg^{-1} RNA. DNA-rich preparations were derived from extraction of fresh tissue with cold phenol and winding high molecular weight DNA from a 2 M LiCl-soluble fraction after addition of three volumes of ethanol. After sedimentation in 1.73 g cm^{-3} CsCl for 64 h at 10°C and 35,000 r.p.m. in a Beckman 65 rotor, the DNA in the supernatant was again removed by winding on to a glass rod after removal of CsCl by dialysis and addition of three volumes of ethanol. Final samples were resuspended and dialysed against TKM buffer (0.01 M Tris, 0.1 M KCl, 10^{-4} M MgCl, pH 7.4). Sample (d) was treated with $15 \mu\text{g ml}^{-1}$ pancreatic DNase I at room temperature for 15 min immediately before electrophoresis¹⁰. Gels were scanned in a Beckman ACTA CII spectrophotometer, sliced on a Mickle model 140 gel slicer, and either incubated for 48 h at 37°C in omnifluor toluene scintillator + 3% protosol before counting, or eluted overnight in distilled H_2O for recovery of nucleic acid species. Samples from gel areas 1, 2, 3, 4, indicated in (c) and (d) were hybridised in 40% formamide with ^{125}I -CEV-RNA according to Friedrich and Feix⁸ with the RNase-resistant c.p.m. as follows: Area 1, 958; 2, 17; 3, 62; 4, 375.

prepared from healthy and infected gynura and tomato (*Lycopersicon esculentum* Mill, cv. Rutgers) as well as CEV-resistant tobacco (*Nicotiana tabacum*) and cowpea (*Vigna unguiculata* (L.) Walp. cv. Early Ramshorn. Table 2 indicates that ^{125}I -CEV-RNA specifically hybridises only with DNA extracted from diseased tissues. The existence of a CEV-like DNA is further supported by the reduction in activity in the presence of added carrier CEV-RNA as well as the dependence of RNase resistant activity on DNA concentration. Pre-treatment with DNase, however, could not completely reduce the count level to background, presumably because of the ability of DNA fragments to hybridise with the ^{125}I -CEV-RNA, as suggested by gel electrophoresis (Fig. 1c, d).

Verification of the presence of a CEV-like DNA was made by analysis of DNA preparations in 5% polyacrylamide electrophoresis and CsCl equilibrium sedimentation. The quality of the isolated CEV-RNA preparation before and after iodination is shown in the gel patterns of Fig. 1a and b. *In vitro* labelling with ^{125}I as reported by Prenskey *et al.*⁹ results in the degradation of a portion of the CEV-RNA population to approximately 2/3 and 1/3 fragments (Fig. 1a). Since DNA preparations were not RNase treated, contaminating RNA species, principally 4S and 5S RNA, are observed even following successive windings from 75% ethanol medium coupled with centrifugation in 1.73 g cm^{-3} CsCl for 65 h.

The position of DNA on the 5% gels (Fig. 1c) was identified by the action of DNase (Fig. 1d). Areas of these gels (indicated in Fig. 1c and d as areas 1, 2, 3, 4) containing the region of native DNA (area 1) and the oligodeoxynucleotides (area 4) were eluted and concentrated. Hybridisation with ^{125}I -CEV-RNA indicated the presence of the CEV-complement in the region of native DNA as well as the DNase-produced product. This further supports the existence of a CEV-like DNA species but does not obviate the possible existence of an RNA moiety trapped in the DNA matrix which is complementary to CEV-RNA and demonstrates a distinctly different mobility in a DNase-treated preparation.

For this reason, DNA preparations were subjected to CsCl equilibrium sedimentation (Fig. 2), and samples recovered from the DNA band as well as any pelleted RNA, were hybridised with ^{125}I -CEV-RNA. A CEV-like DNA is contained in the CsCl banded DNA. No evidence was detected for RNA-RNA hybridisation of CEV-RNA to either pelleted RNA or tRNA-like species concentrated at high CsCl densities. Sedimentation in 56% (w/w) CsCl (Fig. 2) indicated heterogeneity in the DNA preparations from healthy gynura

the host cell, offer striking parallels to the oncogenic viral processes operating at a minimal level.

In an attempt to localise and identify the CEV-RNA template, total nucleic acids extracted from subcellular fractions of infected *Gynura aurantiaca* were hybridised⁸ with iodinated ^{125}I -CEV-RNA⁹ (Table 1). The most significant level of RNase-resistant ^{125}I -CEV-RNA binding was observed with nucleic acid from the 250-g pellet which contained the bulk of the nuclear DNA as judged by the diphenylamine test. This fraction also contained pathogenic CEV-RNA as indicated by the relative 'infectivity' assay.

To determine whether a CEV-like DNA was specific to exocortis disease tissues, high molecular weight DNAs were

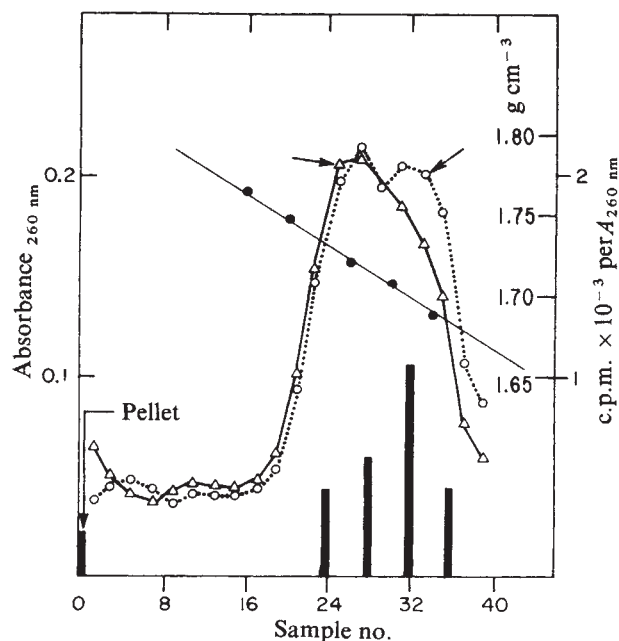


Fig. 2 Equilibrium sedimentation of DNA-rich preparations from healthy and exocortis-infected gynura in 56% (w/w) Cs_2SO_4 and 10°C in the SW 50 Beckman rotor for 65 h in 0.01 M Tris, pH 8.0. The tubes were fractionated by bottom puncturing and 30–40 samples collected from 5-ml columns. Alternate samples were diluted to 1 ml with TKM buffer and $A_{260\text{nm}}$ recorded. Densities were determined on undiluted samples by refractive index. Samples to be used for hybridisation with ^{125}I -CEV-RNA were dialysed overnight against $0.1\times\text{TKM}$ buffer followed by lyophilisation and resuspension in 100 μl H_2O and hybridised as in Fig. 1. The specific activity of c.p.m. per $A_{260\text{nm}}$ (■) records the recovery of RNase-resistant ^{125}I -CEV-RNA in selected samples included sedimented RNA. Δ , Healthy plants; $\circ$, infected.

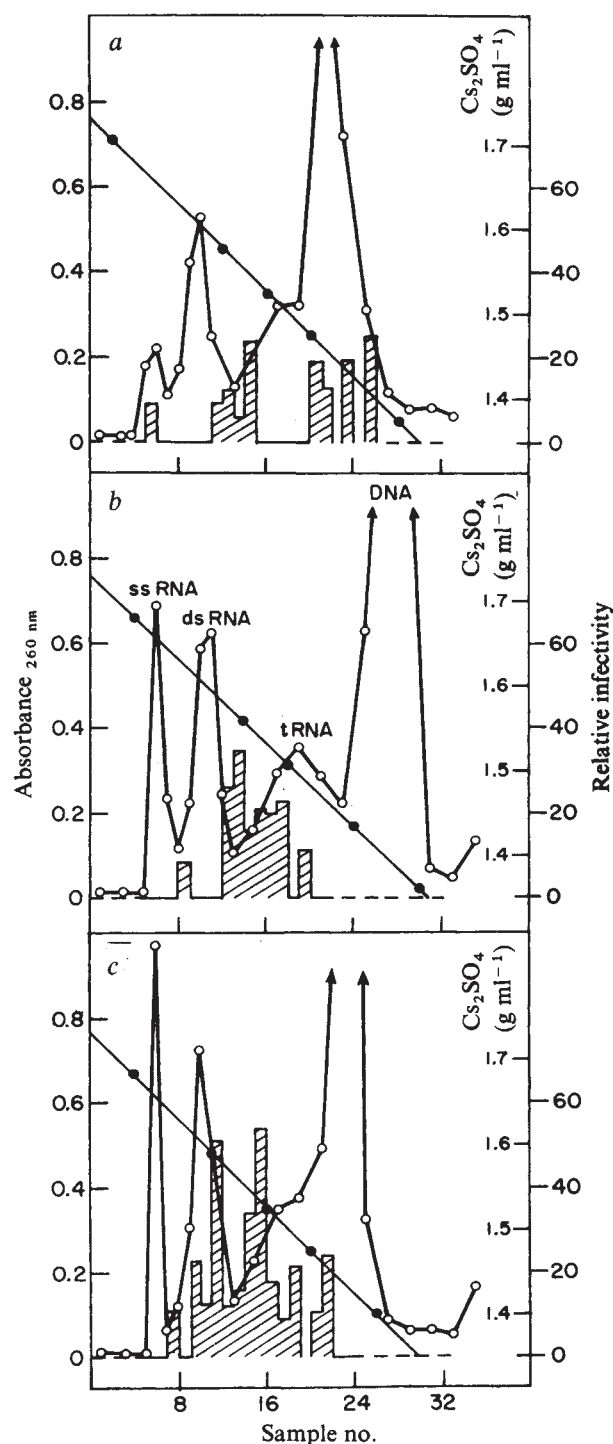


Fig. 3 Sedimentation of DNA-rich preparation from exocortis-infected gynura (b) in 43% (w/w) Cs_2SO_4 , after heating to 100°C for 10 min and slow (a) or rapid cooling (c), in SSC buffer (0.1 M NaCl, 0.01 M sodium citrate, pH 7.0) centrifuged in the Beckman SW 50.1 rotor at 10°C and 30,000 r.p.m. for 65 h. Samples were fractionated and analysed as in Fig. 2 and dialysed against TKM buffer before bioassay. Biological activity was determined by the appearance of stunting and epinasty symptoms on gynura over a 30-d period following inoculation by razor-slashing of the stems. The total number of 'infected-plant-days' on 3–4 gynura plants per sample comprised the relative infectivity¹¹ value (shaded areas). $\circ$, $A_{260\text{nm}}$.

with the presence of a fraction of lower density than the main-band DNA. This light satellite DNA is more easily resolved in DNA preparations from diseased gynura and demonstrates a greater affinity for CEV-RNA than mainband DNA. Since the CEV-RNA is 58% G+C (ref. 2) the putative DNA complement would be correspondingly high in A+T and therefore characterised by relatively low intrinsic buoyant density. Furthermore, the enhanced resolution of the light DNA in preparations from diseased tissue suggests the proportionately higher concentration of the light DNA possibly induced by the pathogenic activity of CEV-RNA.

These data indicate the existence of a DNA sequence in diseased tissue with an affinity for the CEV-RNA resulting in an RNase-resistant RNA-DNA hybrid. Previous studies¹ demonstrated that the pathogenic RNA contained in 2 M LiCl supernatant preparations from exocortis disease tissue could be localised after sedimentation in Cs_2SO_4 in a region intermediate to dsRNA (1.60 g cm^{-3}) and tRNA ($1.51\text{--}1.58\text{ g cm}^{-3}$). If the CEV-RNA became associated with relatively high molecular weight species of DNA then a shift of the pathogenic activity to lower density Cs_2SO_4 might be expected.

Figure 3b shows the separation of single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), tRNA and DNA along with the distribution of CEV-RNA by the relative infectivity index. Aliquots of the sample (Fig. 3b) were subjected to conditions of melting and rapid (Fig. 3c) or slow (Fig. 3a) cooling. A shift in the density of the DNA sample (Fig. 3a, c) from 1.40 g cm^{-3} to 1.44 g cm^{-3} resulted from the melting at 100°C for 10 min. The demonstration of pathogenic activity was not affected by the thermal treatment,

reflecting the unusual stability of the viroid RNA. The relatively normal distribution of relative infectivity in Cs_2SO_4 gradient observed in the control preparation (Fig. 3b), however, was

Table 1 Distribution of hybridisable nucleic acid in subcellular fractions from *Gynura aurantiaca* infected with CEV

Subcellular fraction	RNase resistant* ¹²⁵ I		DNA		Relative infectivity	
	(c.p.m.)	(%)	(µg per 100 µl)	(%)	Index	%
250g for 10 min	3,976	56	76	84	30	39
1,000g for 10 min	913	13	6.8	8	20	26
80,000g for 30 min	860	12	2.8	3	27	35
100,000g for 2 h	792	11	2.4	3	0	0
Supernatant	584	8	2.4	3	0	0

*35 µl of each DNA fraction used for hybridisation treatment. RNase-resistant ¹²⁵I was determined as described in Fig. 1.

altered in both the rapid and slow-cooled treatments to include a broader density range, encompassing the region of DNA. The distribution of the preparation reannealed in slow cooling conditions (Fig. 3a) is particularly striking, since a significant portion of the CEV-RNA now cosediments with the gynura DNA.

The CEV-specific DNA, concentrated in the 250-g pellet (Table 1) supports a nuclear phase in the synthesis of viroid RNA¹². This is not to imply that the pathogenic RNA is totally restricted to the host nucleus¹³. Whether the DNA complementary to CEV-RNA is synthesised *de novo* by the action of an induced RNA-directed DNA polymerase, or exists in a limited number of copies and is amplified by normal DNA polymerase activity in the process of pathogenesis, remains to be determined. The mode of synthesis of CEV-RNA as well as the mechanism involved in the production of the pathological condition is critical to the description of the

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Table 2 Hybridisation of ¹²⁵I-CEV-RNA to various DNA-rich preparations

DNA Source	RNase-resistant ¹²⁵ I (c.p.m.)
Tobacco	70
Cowpea	0
Tomato	233
Gynura 5 µg	133
Gynura 10 µg	116
CEV-infected tomato	1,731
CEV-infected tomato + 1.6 µg CEV-RNA	170
CEV-infected tomato + DNase*	350
CEV-infected gynura 5 µg	1,105
CEV-infected gynura 10 µg	1,465
CEV-infected gynura + 1.6 µg CEV-RNA	153
CEV-infected gynura + DNase*	859

RNase-resistant ¹²⁵I was determined after hybridisation of 0.005 µg ¹²⁵I-CEV (50,000 c.p.m.) with 10 µg (except as indicated) of the DNA preparation as described in Fig. 1.

*Incubated 0.75 h at room temperature with 1 µg DNase I in a reaction volume of 40 µl.

unique class of pathogenic RNA, the viroids. The CEV-RNA reflects neither the structural² nor the biological properties^{3,4} of most plant viral nucleic acids. This report further supports a unique role for the viroid RNA as a regulator of the expression of the normal host genome resulting in the production of a 'disease-like' symptom. The implied participation of a DNA intermediate reported here as well as the observed requirement for cell division in the synthesis of CEV-RNA (J.S.S., unpublished) further supports the intimate interaction between the viroid RNA and host cell, resulting in a condition analogous to cell transformation.

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Presence of sequences hybridisable to dsRNA in cytoplasmic mRNA molecules

In recent years, a precursor-product relationship between heterogeneous nuclear RNA (HnRNA) and cytoplasmic messenger RNA (mRNA) has been investigated intensively^{1,2}. The HnRNA was shown to have a double-stranded hairpin structure within its long molecules³⁻⁵. This particular region has been isolated as a double-stranded RNA (dsRNA) after treatment with ribonucleases, followed by purification on cellulose⁵ or hydroxyapatite^{3,4}. The HnRNA of higher organisms is degraded largely in the nucleus and, at most, 10% of the nuclear content

is transported to the cytoplasm². It remains unknown whether the double-stranded structures in the HnRNA molecules are totally degraded in the nucleus or whether sequences involved in this structure are preserved in part or *in toto* during nuclear processing and possibly become parts of cytoplasmic mRNA.

We investigated the possibility that sequences from double-stranded structures are present in cytoplasmic mRNA molecules and present data showing that there are sequences hybridisable to denatured dsRNA in mRNA molecules and that these sequences are 6.1% of the total mRNA. We also tried to investigate the relative position of these sequences in mRNA molecules using 5'-exonuclease and polynucleotide phosphorylase.

The experiment consists of the molecular hybridisation between labelled mRNA and unlabelled dsRNA, both prepared from rat livers. To estimate the percentage hybridisation of mRNA to dsRNA, purified mRNA was annealed to dsRNA at varying concentrations, but at a fixed input ratio (dsRNA:mRNA, w/w) of 1:1. This input ratio was chosen since hybrids formed at low input ratio, that is, 1:1, exhibited a sharp melting profile with a T_m only 6 °C below that of the original dsRNA (ref. 6), indicating a reasonably good fidelity of base pairing.

mRNA hybridised to dsRNA on annealing (Fig. 1) and the hybridisation reaches a plateau at a value of approximately 30 mg ml⁻¹ h⁻¹. Since contamination of nuclear RNA, including dsRNA, in the mRNA preparation was extremely low⁶, this observation did not result from the hybridisation of labelled dsRNA (contaminating the labelled mRNA preparation) to the added dsRNA, but certainly represents the hybrid formation between labelled mRNA and dsRNA added. The hybridisation curve shows that dsRNA hybridised to 6.1% of mRNA added. Assuming that the average value for the length of an mRNA molecule is approximately 1,000 nucleotides¹¹, then the total length of the sequences involved would be approximately 60 nucleotides. This is within the size range of purified rat liver dsRNA, as estimated by electrophoresis on 10% acrylamide gels⁵ and by electron microscopy according to the aqueous technique of David *et al.*¹². The purified dsRNA showed a broad size distribution.

This observation is of particular importance with respect to the hypothesis that HnRNA is a precursor of cytoplasmic mRNA (ref. 13). It has been demonstrated that all dsRNA is of nuclear origin and is not derived from ribosomal, preribosomal and transfer RNAs (refs 3–5). In particular, dsRNA obtained from high molecular weight nuclear RNA is in the form of a hairpin structure in the HnRNA molecule with a linking nucleotide sequence at the top of the hairpin (refs 3, 4 and R. P. Monckton and H. N., unpublished). No definite double-stranded structure has been detected in the cytoplasm⁵ or in the cytoplasmic mRNA^{3,6}. Electron microscopic observations of isolated mRNA molecules did not reveal any double-stranded structure either. Although a secondary structure for particular species of eukaryotic mRNA has been suggested after examining amino acid sequences^{14,15} and performing hyperchromicity experiments¹⁶, no conclusive evidence has yet been reported. In addition, denatured dsRNA prepared from nuclear RNA and completely free of cytoplasmic mRNA also hybridised to mRNA. Therefore, the presence of sequences in the mRNA molecule hybridisable to dsRNA indicates that, if no post-transcriptional addition of these sequences to mRNA occurs, mRNA is most likely to be derived from sequences contained in HnRNA. The absence of actual double-stranded structures in mRNA is presumably because only a portion of the sequences necessary for double-stranded hairpin structuring is retained during the processing of HnRNA to mRNA. There is considerable evidence supporting the hypothesis of a precursor-product relationship of HnRNA and mRNA. What doubt there is arises mainly from the fact that most of the experiments did not completely eliminate the possibility of the presence of cytoplasmic mRNA contaminating the HnRNA preparation^{17,18}. Our present observations support the possible precursor-product relationship between HnRNA and mRNA

without any doubt of contamination by mRNA, since purified dsRNA is derived from HnRNA and is free of cytoplasmic mRNA (ref. 5).

We then investigated whether sequences hybridisable to dsRNA are located at or near the 5'- and (or) 3'-end of the cytoplasmic mRNA molecule. The hybrids between dsRNA

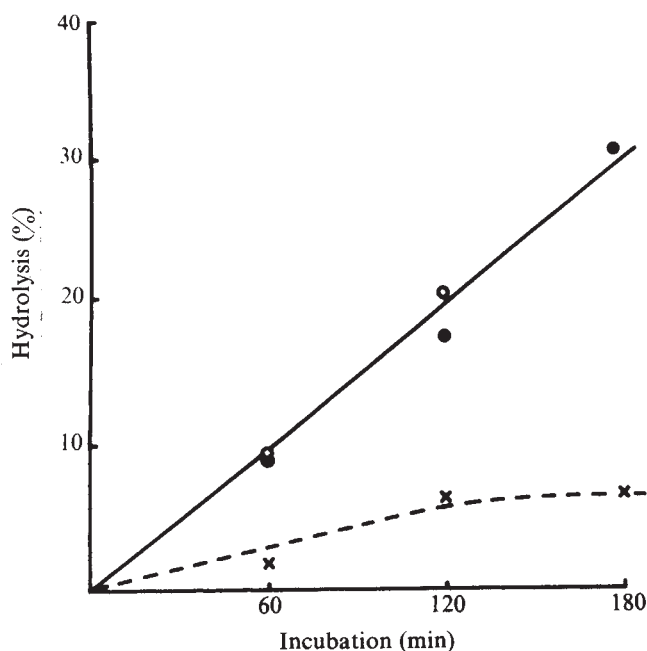


Fig. 1 Hybridisation of ³H-labelled mRNA to unlabelled dsRNA. For preparation of ³H-labelled mRNA, ³H-orotic acid (300 μCi) was injected into rat portal vein 4 h before decapitation. Polyosomes were obtained from labelled livers according to the method of Schreier and Staehelin⁷ and then polysomal RNA was extracted by treatment with chloroform-phenol⁸. mRNA was isolated from polysomal RNA by sucrose density gradient centrifugation as described previously⁶. Our mRNA preparation has already been characterised and exhibited messenger activity in a cell-free protein-synthesising system⁶. dsRNA was prepared and purified from rat livers as previously⁵. The preparation procedure involved extensive treatment of whole cell or nuclear RNA with DNase, Pronase and RNases (A and T₁). The crude preparation of dsRNA was purified on a cellulose column. The dsRNA preparation was completely free of DNA⁵. All hybridisation reactions were carried out at 72 °C for 24 h in hybridisation solution (10 mM phosphate buffer, pH 7.6, 0.3 M NaCl) by a modification of Schonberg *et al.*⁹ and Shimotohno and Miura¹⁰. Larger values of mg ml⁻¹ h⁻¹ were obtained by increasing the concentration of RNA and not by lengthening the reaction time. After annealing, the reaction mixture was diluted to a final volume of 0.2 ml with hybridisation solution and subjected to treatment with 10 μg ml⁻¹ of RNase A and 0.5 μg ml⁻¹ of RNase T₁ at 37 °C for 30 min. It was shown that the RNase-resistance observed after annealing certainly results from hybrid formation⁶. The dsRNA-mRNA hybrids formed were precipitated in the presence of carrier RNA with trichloroacetic acid, collected on glass fibre filters and the radioactivity determined⁵. Since 0.6% of mRNA became RNase resistant after annealing without dsRNA, this 'blank' value was subtracted from the measured values. The RNase-resistant materials obtained after annealing without dsRNA did not behave as typical double-stranded structures, since this resistance to RNases was clearly not lost after thermal denaturation.

and labelled mRNA were treated with either spleen phosphodiesterase (with or without added phosphomonoesterase) or polynucleotide phosphorylase. The secondary structure of polynucleotides is insensitive to these enzymes, which attack

only single-stranded polynucleotides from the 5'-end (spleen phosphodiesterase¹⁹) or 3'-end (polynucleotide phosphorylase²⁰) of the molecule.

Treatment of ³H-labelled heat-denatured hybrids or cytoplasmic mRNA alone with spleen phosphodiesterase in the conditions described in Fig. 2 released radioactivity almost linearly with incubation time (Fig. 2). Hydrolysis of the intact hybrids took place at a much slower rate in the same conditions during incubation for 60–180 min. Since commercial preparations of spleen phosphodiesterase contain contaminating

between intact and denatured hybrids suggests that sequences hybridisable to dsRNA do not occur in clusters along the entire molecule, as may be the case in a special class of RNA which would be a small proportion of the mRNA population, and that most of the mRNA molecules possess sequences hybridisable to dsRNA. This is indeed the case, since hybrids were formed between dsRNA and 98% of the ³H-labelled mRNA population as evidenced by the retention of ribonuclease-untreated hybrids on a hydroxyapatite column. The mRNA molecule seems likely to be composed partly of sequences

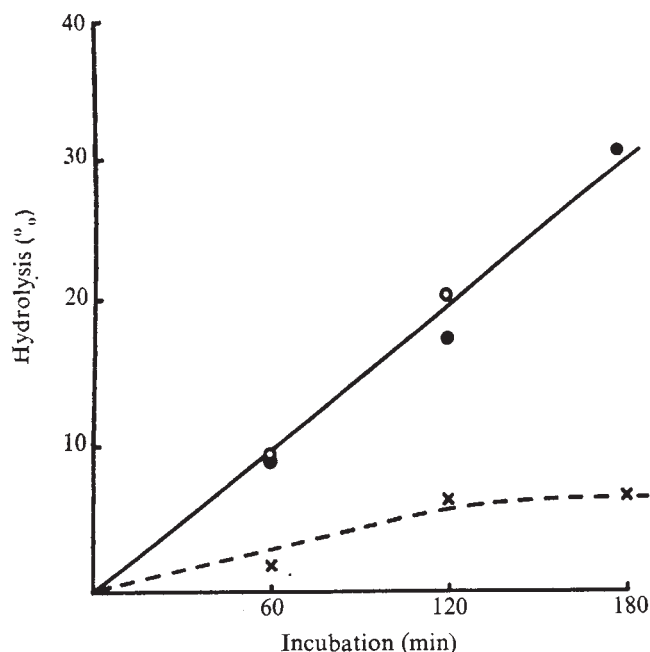


Fig. 2 Hydrolysis of dsRNA-mRNA hybrids by spleen phosphodiesterase. The hybrids formed with denatured dsRNA and ³H-labelled mRNA (approximately 1,000–1,500 c.p.m.) at an input ratio of 1:1 at a value of 10–15 mg ml⁻¹ h⁻¹ were treated at 37 °C with 250 µg ml⁻¹ of spleen phosphodiesterase (Worthington Biochem. Corp.) in hydrolysis solution (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 0.3 M NaCl) based on the conditions used for reducing the activity of contaminating endonucleases²¹. Phosphodiesterase activity still remains at high pH (ref. 22). The hybrid preparations used here were not purified after annealing and so contained some residual unhybridised mRNA. After incubation, the samples were chilled in ice and trichloroacetic acid was added to terminate the reaction. The radioactivity of the acid-insoluble residue was measured as described in Fig. 1. Heat-denatured (100 °C for 10 min, followed by immediate cooling in ice) hybrids (●) were used as a control for comparison of the susceptibility of intact hybrids (×). Hydrolysis of mRNA alone (○) was also monitored. This occurred in exactly the same fashion as that of denatured hybrids.

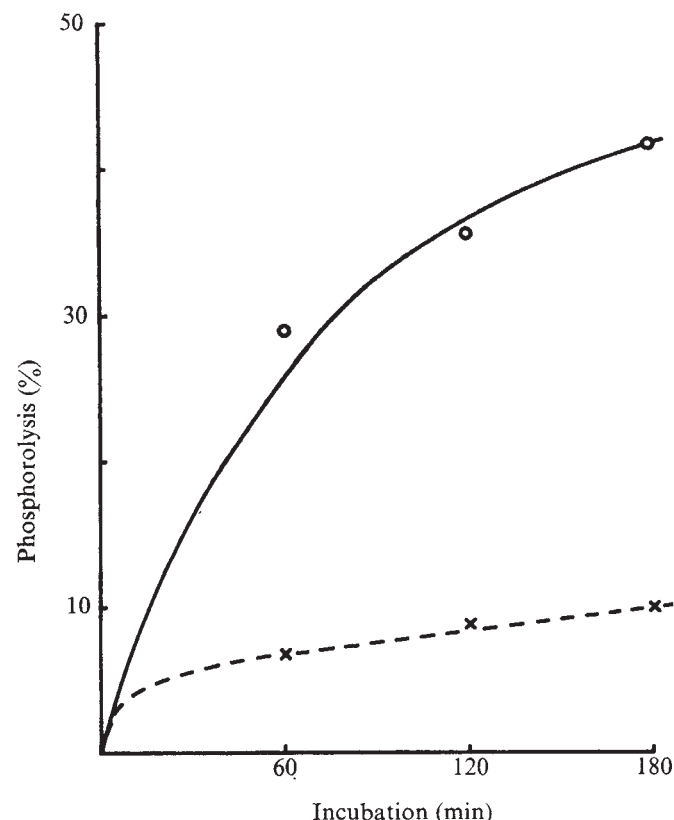


Fig. 3 Phosphorolysis of dsRNA-mRNA hybrids by polynucleotide phosphorylase. The hybrids formed in the conditions described in Fig. 2 were treated at 37 °C with 125 units ml⁻¹ of polynucleotide phosphorylase (*Micrococcus lysodeikticus*, Sigma Chemical Co.) in the presence of 1 M NaCl under the conditions described by Soreq *et al.*²⁴. After incubation, the samples were treated as described in Fig. 2. ○, Heat-denatured; ×, intact hybrids.

phosphomonoesterase activities²³, addition of phosphomonoesterase (0.45–1.7 × 10⁻³ units per µg RNA, Sigma Chemical Co.) to the incubation mixture resulted in only a 10–15% increase in hydrolysis. The low susceptibility of the intact hybrids to spleen phosphodiesterase suggests that there are almost no long single-stranded tails on the 5'-end of the hybrids. This suggests the presence of sequences hybridisable to dsRNA at or near the 5'-end of mRNA molecules. The possibility that mRNAs contain these sequences also at or near the 3'-end cannot, however, be excluded. In fact, intact hybrids were also less susceptible than denatured hybrids to degradation from the 3'-end by polynucleotide phosphorylase (Fig. 3).

The remarkable difference in susceptibility to these enzymes

hybridisable to dsRNA, partly of a unique sequence coding for polypeptide and partly of a poly(A) segment. The results obtained here reveal nothing of the presence or absence of sequences hybridisable to dsRNA in the internal positions of the mRNA molecule; however, since a major portion of the mRNA molecule is the unique sequence coding for polypeptide, sequences hybridisable to dsRNA are unlikely to be in the middle of the molecule. Such sequences would seem to be at or near the 5'-end and also between the unique sequence and the poly(A) segment at the 3'-end. There is evidence suggesting the presence of these sequences at or near the 5'- and 3'-ends of the mRNA molecules. First, sequences transcribed from repetitious DNA sequences, from which dsRNA is transcribed, have been detected at or near the potential 5'-end of the mRNA portion of HnRNA molecules²⁵. Second, repetitious sequences were actually found at the 5'-end of *Xenopus* embryo mRNA²⁶, and both at the 5'- and 3'-ends of slime mould mRNA and nuclear RNA (ref. 27).

Taking all these facts into account, it is suggested that with the exception of some mRNAs, cytoplasmic mRNA is at least

composed of a sequence corresponding to a portion of one strand of dsRNA both at, or near, the 5'-end and probably between the unique sequence coding for polypeptide and the poly(A) segment at the 3'-end. The existence of sequences hybridisable to dsRNA is consistent with the hypothesis that the dsRNA hairpin structure of the HnRNA molecule is a cleavage point which may be specifically recognised by a nuclear processing enzyme. This implies that the poly(A) segment linked to the unprocessed HnRNA molecule would be detached during the maturation process and a new poly(A) segment would be added to the 3'-end after completion of the processing. Recent experiments have revealed that much of the poly(A) segment attached to nuclear RNA decays in the nucleus and is not conserved^{2,28}. None of the experiments carried out so far on polyadenylation excludes the possibility that poly(A) may be added to new 3'-ends generated by the processing of HnRNA as well as to the 3'-end of the unprocessed HnRNA molecule². Moreover, it seems likely that addition of a poly(A) segment takes place both before and after nuclear processing of HnRNA molecules²⁹.

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CO₂ to Hb takes place by a reaction of CO₂ with the α -amino groups to form carbamino compounds^{4,5}, and binding curves can be measured by rather laborious methods^{4,6}. Perrella *et al.*⁷ have modified these methods so that much smaller amounts of Hb, such as the specifically carbamylated Hbs⁸, can be used. In this method, Hb equilibrated with CO₂ is rapidly taken to pH 11 to stabilise the carbamino CO₂ and BioRad AG 1 $\times$ 8 resin added to remove carbonate and bicarbonate ions. The carbamino CO₂ is displaced from the Hb by acidification and measured in a Van Slyke apparatus. DPG-free human deoxyhaemoglobin gave diphasic CO₂ binding curves⁷ showing that the affinities of the α and β chain α -amino groups for CO₂ are different, but it was impossible to establish which group had the higher affinity.

We have used the specifically carbamylated Hbs, $\alpha_2\beta_2$ and $\alpha_2\beta_2^c$ where c denotes specific reaction of the α -amino group with cyanate⁸, to identify the low and high affinity sites for CO₂ in Hb.

As a control experiment we first measured the CO₂ bound to $\alpha_2\beta_2^c$; this amount was very small but not negligible and was about 10% of that bound to normal Hb in the same

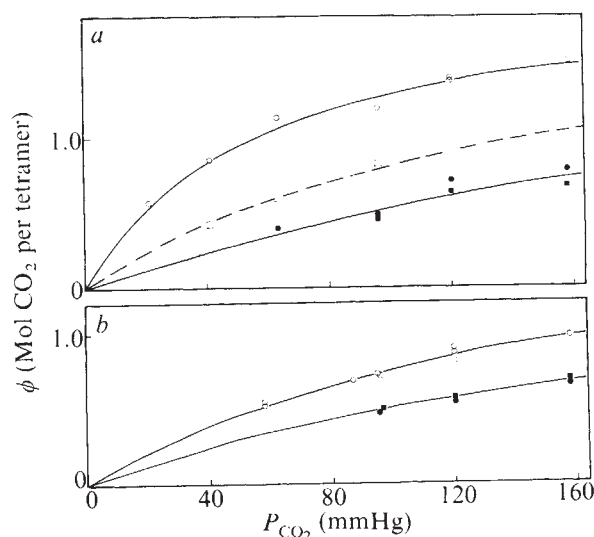


Fig. 1 CO₂ bound by $\alpha_2\beta_2$ (a) and $\alpha_2\beta_2^c$ (b) at pH 7.4, 37 °C. Haemoglobin concentration 3.5 mM in tetramer, ionic strength 0.18. $\circ$, Deoxy; $\square$, deoxy + 2 mol DPG per tetramer; $\bullet$, CO; $\blacksquare$, CO + 2 mol DPG per tetramer. The data were corrected for a 5% contamination with normal Hb. The P_{CO_2} can be converted to mM CO₂ by multiplying the value of mmHg by 0.031. The solid lines through the points are the calculated curves with values of the pH-dependent association constants λ of $\lambda_\beta = 579 \text{ M}^{-1}$ and $\lambda_\beta^{CO} = 120 \text{ M}^{-1}$ for deoxy and CO $\alpha_2\beta_2$ and $\lambda_\alpha = 190 \text{ M}^{-1}$ and $\lambda_\alpha^{CO} = 100 \text{ M}^{-1}$ for deoxy and CO $\alpha_2\beta_2^c$. The broken line is the curve calculated from equation (1) (using a program written by Dr A. P. Minton) with $K_p = 5 \times 10^3 \text{ M}^{-1}$, $K_p' = 1,700 \text{ M}^{-1}$, and $K_p'' = 500 \text{ M}^{-1}$.

Identification of the high and low affinity CO₂-binding sites of human haemoglobin

THE unloading of oxygen in the tissues is facilitated by lowering the oxygen affinity of haemoglobin (Hb) by both CO₂ and diphosphoglycerate (DPG)¹. This occurs because CO₂ and DPG are oxygen-linked, that is, they are bound more firmly to deoxyhaemoglobin than to oxyhaemoglobin^{2,3}. The binding of

conditions. This small amount of oxygen-linked CO₂ could be bound to a site different from the α -amino groups or could be the result of about 10% contaminating normal Hb. The CO₂ bound to $\alpha_2\beta_2$ and $\alpha_2\beta_2^c$ is shown in Fig. 1.

In the DPG-free deoxy forms $\alpha_2\beta_2$ binds more CO₂ than $\alpha_2\beta_2^c$, while in the CO forms both bind CO₂ equally. The calculated pH-dependent association constants (Fig. 1) show that in the deoxy form the β chain α -amino group has about a threefold higher affinity for CO₂ than the α chain α -amino group. This is about the same difference found between the

two unidentified binding sites in normal human deoxyhaemoglobin⁷, and is in agreement with the effect of CO₂ on the oxygen affinity of $\alpha_2\beta_2$ and $\alpha_2\beta_2^c$ (ref. 8).

Figure 2 shows that the addition of the CO₂ bound by $\alpha_2\beta_2$ and $\alpha_2\beta_2^c$ is nearly the same as the total CO₂ bound by normal Hb. This indicates that the free α -amino groups in $\alpha_2\beta_2$ and $\alpha_2\beta_2^c$ bind the same amount of CO₂ as the same groups in normal Hb and these derivatives can thus be considered good model compounds for the study of normal Hb.

DPG does not affect the CO₂ binding of deoxy $\alpha_2\beta_2^c$ or the CO forms of $\alpha_2\beta_2^c$ and $\alpha_2\beta_2$, but reduces the CO₂ binding of deoxy $\alpha_2\beta_2$ by about 30% (Fig. 1), in agreement with the fact that DPG interacts with the β chain α -amino group of deoxy-

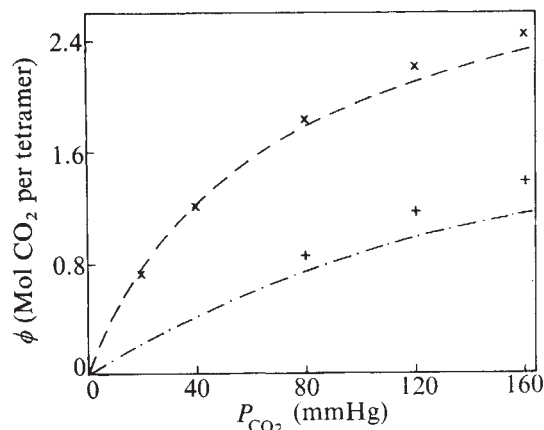
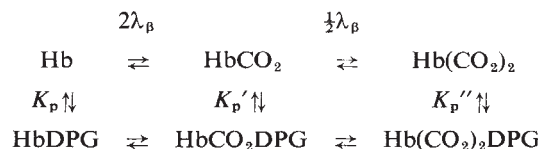


Fig. 2 CO₂ bound by normal deoxyhaemoglobin (---) and CO-haemoglobin (---) in the same conditions as Fig. 1, taken from ref. 7. The symbols are the sum of the two sets of CO₂ binding data for deoxy (x) and CO (+) $\alpha_2\beta_2^c$ and $\alpha_2\beta_2$ taken from Fig. 1.

haemoglobin⁹. The total CO₂ bound to $\alpha_2\beta_2$ and $\alpha_2\beta_2^c$ in the presence of DPG equals that bound to normal deoxyhaemoglobin in the same conditions. Thus at 42 mmHg P_{CO_2} , deoxy $\alpha_2\beta_2$ and $\alpha_2\beta_2^c$ bind, in total, 0.64 mol CO₂ per tetramer (Fig. 1) compared with the 0.68 mol CO₂ per tetramer found, in this study, bound to normal deoxyhaemoglobin. A similar reduction in CO₂ binding by DPG or ATP was found by Brenna *et al.*¹.

X-ray crystallography shows that binding of two CO₂ molecules to the β chain α -amino groups¹⁰ would decrease the binding of DPG, but it is very difficult to make a quantitative estimate from these data. The CO₂ binding data of deoxy $\alpha_2\beta_2$ in the presence of DPG can be used to estimate values of K_p and K_p'' (the DPG association constants when one or two CO₂ molecules are bound) provided that the value of K_p (the DPG association constant of $\alpha_2\beta_2$ in the absence of DPG) is known. We have used $\alpha_2\beta_2$ rather than normal Hb because complications caused by CO₂ binding to the α chain α -amino group are avoided. The K_p values measured in conditions similar to ours (that is, concentrated deoxyhaemoglobin at 37 °C) are 10^4 M⁻¹ (ref. 11) and 5×10^3 M⁻¹ (ref. 12) at pH 7.2 and ionic strength 0.12 and 0.15, respectively. Our conditions of pH 7.4 and ionic strength 0.18 should decrease these values only slightly³. The K_p for $\alpha_2\beta_2$ is likely to be the same as for deoxyhaemoglobin because DPG causes the same change in log P_{50} as in normal Hb⁸. This shows that the difference between the DPG association constants of the oxy- and deoxy-forms of $\alpha_2\beta_2$ are the same as for normal Hb, and it is likely that their absolute values are the same.

If DPG binds to only one site in deoxyhaemoglobin and CO₂ and DPG can simultaneously bind at this site, then the data can be analysed by the following scheme



where Hb represents $\alpha_2\beta_2$, λ_β is the association constant for CO₂ binding to the β chain α -amino group⁷, K_p is the association constant for DPG binding to deoxy $\alpha_2\beta_2$ in the absence of CO₂, and K_p' and K_p'' the DPG association constants when one CO₂ or two CO₂ molecules are bound. The number of mol per tetramer of CO₂ (ϕ) bound to the β chain α -amino group in the presence of DPG, is

$$\phi = \frac{2\lambda_\beta[\text{CO}_2](1 + \lambda_\beta[\text{CO}_2] + K_p[\text{DPG}] + \lambda[\text{CO}_2]K_p''[\text{DPG}])}{(1 + \lambda_\beta[\text{CO}_2])^2 + K_p[\text{DPG}] + 2\lambda_\beta[\text{CO}_2]K_p'[\text{DPG}] + \lambda_\beta^2[\text{CO}_2]^2K_p''[\text{DPG}]} \quad (1)$$

The value of K_p in equation (1) can be determined when the ratio K_p/K_p'' is known. When K_p/K_p'' is less than 10, then large values of K_p ($> 2 \times 10^4$ M⁻¹) will fit the data in Fig. 1. As K_p/K_p'' is progressively increased, then K_p must be decreased to fit the data. Thus, if $K_p/K_p'' > 100$ then K_p must be less than 10^3 M⁻¹, a value clearly too small in respect to the directly-measured values of K_p (refs 11, 12). Figure 1 shows the fit obtained with $K_p/K_p'' = 10$ and K_p set at 5×10^3 M⁻¹ to comply with the directly-measured values^{11,12}. This low ratio of K_p/K_p'' shows that substantial populations of the species HbCO₂DPG and Hb(CO₂)₂DPG must exist.

Brenna *et al.*¹ attempted to identify the high affinity CO₂ binding site from the CO₂ binding data of normal deoxyhaemoglobin in the presence of ATP or DPG. They used $K_p = 10^4$ M⁻¹ but erroneously assumed, in absence of crystallographic data, K_p' and K_p'' to be zero. When these values of K_p , K_p' , and K_p'' are used in equation (1) a low value of λ_β is calculated, suggesting that the high affinity CO₂ binding site in deoxyhaemoglobin is the α chain α -amino group. Clearly our finding of substantial populations of HbCO₂DPG and Hb(CO₂)₂DPG species invalidates the simplified analysis of Brenna *et al.*¹.

Our data are partly in agreement with the crystallographic work of Arnone¹⁰ who found clear evidence of CO₂ binding to the β chain α -amino group but no CO₂ on the α chain α -amino groups and instead CO₂ bound to the interior of the protein near the β chain haem group. It is not clear whether this CO₂ would still be bound in solution in the red cell or whether it would be oxygen linked. The failure to observe CO₂ binding to the α chain α -amino group is surprising because $\alpha_2\beta_2^c$ clearly binds CO₂, which is practically abolished when its free α -amino group is blocked in $\alpha_2\beta_2^c$.

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Spectral changes and allosteric transition in trout haemoglobin

PREVIOUS results have shown that two of the haemoglobin components from trout blood, Hb trout I and Hb trout IV, have different functional properties, which may be correlated directly with their physiological role (see ref. 1 for review). Thus, Hb trout I, which displays cooperative ligand binding at all pH values, completely lacks heterotropic interactions; on the other hand, these interactions are clearly evident in Hb trout IV, for which lowering of pH, or addition of organic phosphates, produces a large decrease in oxygen affinity and in cooperativity (n drops from 2.3 at pH 8 to ~ 1 at pH 6)^{2,3}.

Although the molecular interpretation of the Root effect in Hb trout IV is certainly complex, it is probably related to the stabilisation, induced by protons, of a "low affinity" conformational state of the molecule, according to a simplified "two states" model¹. Such evidence has been provided through EPR experiments on the NO-derivative of Hb trout IV (ref. 4). Here we report further evidence that protons and inositol hexaphosphate (IHP) can induce characteristic shifts in the absorption spectrum of the carbon monoxide derivative of Hb trout IV.

Haemoglobin components from trout blood (from *Salmo irideus*) were purified as described previously². All reagents were analytical grade and were used without further purification. Absolute and difference spectra were recorded with a Cary 14 spectrophotometer thermostated at 20 °C.

The Soret absorption band of Hb trout IV fully saturated with carbon monoxide is redshifted by about 1 nm going from pH 8 to 6, as shown by the difference spectra reported in Fig. 1a. Control experiments have shown clearly that the observed difference spectra cannot be attributed to the presence of methaemoglobin contaminations, or to changes in the saturation with CO. In addition it should be emphasised that no pH-dependent difference spectra of comparable magnitude have been detected for the deoxygenated derivative of Hb trout IV, or for Hb trout I both in the carbomonoxy- and in the deoxy-form.

Figure 1b points out that somewhat similar difference spectra are obtained, at constant pH, by addition of IHP. The effect of the organic phosphate seems, however, to be more complex, as different isosbestic points were observed at low and high pH

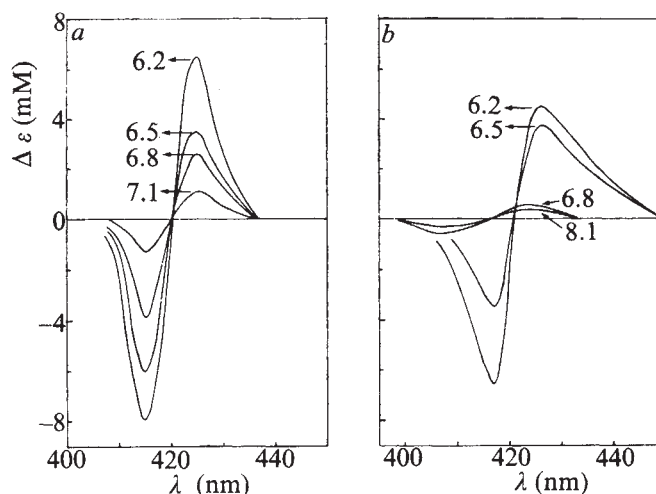


Fig. 1 Effect of pH and IHP on the spectrum of carbomonoxy-haemoglobin trout IV in 0.05 M bis-Tris at 20 °C. a, Difference absorption spectra at various pH, as indicated (reference cell, pH 8.1). b, Difference absorption spectra $\pm 10^{-3}$ M IHP at constant pH, as indicated. Protein concentration 6 μ M (in haem).

values. The results obtained at constant proton activity indicate, in agreement with the functional data⁵, an additional stabilising effect of IHP on the "low affinity" form of liganded Hb trout IV. The titration of Hb trout IV-CO with IHP is shown in Fig. 2. It corresponds to a simple hyperbola, with a value for the overall affinity constant (K) of 1.4×10^4 M⁻¹ at pH 6.2 and

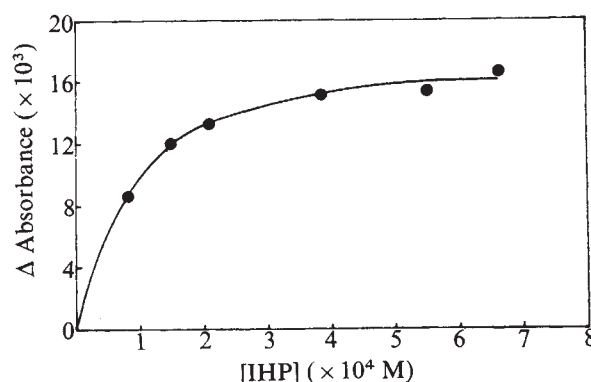


Fig. 2 Binding curve of IHP to Hb trout IV-CO in 0.05 M bis-Tris at 20 °C. Δ Absorbance are the amplitudes of the difference spectra obtained in the presence of increasing amounts of IHP.

20 °C. This value is considerably smaller than that reported for oxygenated human haemoglobin ($K \approx 10^6$ M⁻¹ at pH 7.0 and room temperature)⁶. The difference, although puzzling, is not inconsistent with the known changes in some of the amino acid residues involved in the interactions with IHP within the central cavity of the tetramer⁷.

The existence of isosbestic points, at 420 and 437 nm, in the pH-dependent difference spectra, is in agreement with the presence of only two spectral species in pH-dependent equilibrium. The experimental data in *bis*-Tris (0.05 M) can be fitted with a simple titration curve, with pK 6.5 (Fig. 3), suggesting that only one type of ionisable group is involved in the Root effect. In the presence of IHP (10^{-3} M) a similar effect is observed

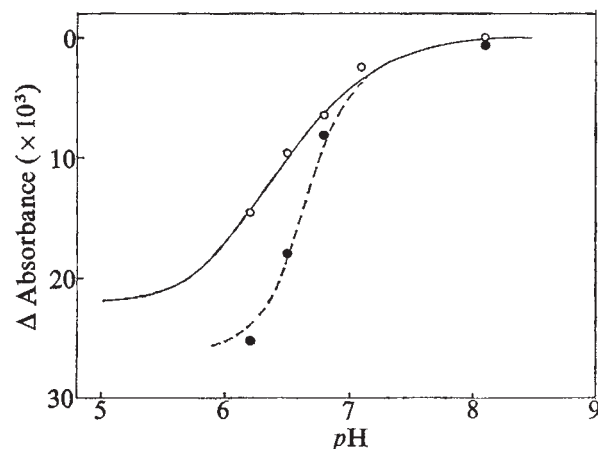


Fig. 3 pH-induced transition in Hb trout IV-CO in the absence (○) and in the presence (●) of 10^{-3} M IHP (solvent 0.05 M *bis*-Tris buffer) at 20 °C; Δ Absorbance as in Fig. 2.

(Fig. 3); the higher pK of the transition is consistent with the cumulative effect that both protons and the organic phosphate exert on the stabilisation of the "low affinity" form. It cannot be excluded, however, that in the presence and absence of IHP the proton induced transition may occur among different states. The greater steepness of the transition observed in IHP is probably related to the fact that Hb trout IV is not fully saturated with the phosphate at all pH values.

It is difficult to interpret the redshift of the maximum of absorption of the CO derivative of Hb trout IV, induced by lowering pH. For instance, it is not possible to correlate unequivocally the position of the peak to the strength of the bond between the iron and the ligand, although obviously the observed changes must reflect a perturbation in the distribution of electrons in the porphyrin-metal-ligand complex. The observed redshift may suggest a weakening of the π -bonding and an increase in the iron-ligand bond length correlated to a movement of the proximal imidazole relative to the metal (R. J. P. Williams, personal communication). Apart from detailed interpretations of the observed effect, it is of interest to correlate the spectral change with other properties of Hb trout IV, which are also pH dependent and occur over the same pH region. First, the dramatic change in functional properties and in particular the well known drop in ligand affinity which is experienced between pH 8 and 5. It seems especially relevant to remark that such a drop in affinity, common to fish haemoglobins, can be largely attributed to a great increase in the dissociation velocity constant of the ligand⁸, which in itself is an indication of a destabilisation of the ligand-protein complex. Second, the fact that pH-dependent ultraviolet difference spectra of Hb trout IV saturated with CO have been observed (M. F. Perutz, personal communication and unpublished data from this laboratory). These difference spectra show a characteristic "fine structure", centred between 280 and 290 nm, which in the case of human haemoglobin has been directly correlated

with the ligand-induced change in the quaternary structure of haemoglobin, and thus should reflect the allosteric conformational transition⁹.

By correlating this information, it seems reasonable to propose that the spectral changes we have reported are themselves an indication of the pH-induced structural transition which, according to the simple model outlined above, is expected to occur in liganded Hb trout IV. This being the case, we may be provided with a method to test possible correlations between the spin state of the molecule and its quaternary structure, and to follow the dynamics of the allosteric transition in Hb trout IV. Experiments along these lines are in progress.

We thank Mr S. Polzoni for this help in preparing the purified Hb components, and R. J. P. Williams for suggesting the possible interpretation of the spectral shift.

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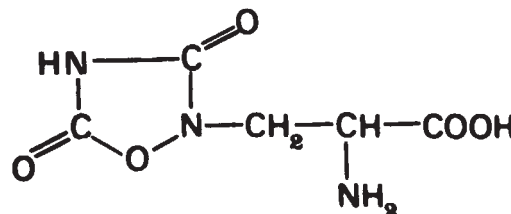
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Corrigendum

In the article "Domoic and quisqualic acids as potent amino acid excitants of frog and rat spinal neurones" by T. J. Biscoe, R. H. Evans, P. M. Headley, M. Martin and J. C. Watkins (*Nature*, **255**, 166-167; 1975) the structural formula given for quisqualic acid in Fig. 1d and Table 2 is incorrect. The structure proposed by Takemoto *et al.* (Takemoto, T., Nakajima, T., Arihara, S., and Koike, K., *Yakagaku Zasshi*, **95**, 326-322 (1975) and **95**, 448-452 (1975) is reproduced below.

In paragraph 2, line 11, the word isoxazolidinedione should read dioxo-oxadiazolidine.



reviews

In the diversity of today's astrophysics, where does radio astronomy stand? That question arises when reading the book by the staff of the National Radio Astronomy Observatory (NRAO) in the United States, a book whose title aims without restriction at the entire Universe beyond the Solar System. One would expect it, then, to cover the vast terrain of astrophysical interactions and phenomena, as seen in the radio window's perspective. A survey of the approximately 1,000 entries in the subject index confirms that indeed an immense variety of objects and processes are touched upon. There are 12 chapters each by a single author; the only chapter on technique *per se*, on "Interferometry and Aperture Synthesis", was written by a pair of authors, synthesising expertise nurtured at Caltech and Cambridge, respectively. It is a wise choice of the editors and a valuable contribution by these authors, providing so clear an exposition of what is the outstanding observation technique at radio wavelengths whenever imaging elements smaller than one arc minute, positional accuracy better than one arc second, and sensitivities per beam lower than a few milli Jansky are called for. Having begun using 1–5-km arrays so successfully at decimetre and centimetre wavelengths, this extremely versatile technique that so thoroughly exploits current computer capacities, is spreading to millimetre-wavelength astronomy and to trans-continental interferometry. The chapter on observation (and reduction) technique conveniently separates the first nine

Bridging the gap in radio astronomy

H. van der Laan

Galactic and Extra-Galactic Radio Astronomy. By the Staff of the National Radio Astronomy Observatory. Edited by G. L. Verschuur and K. I. Kellermann. Pp.x+402. (Springer: Berlin and New York, 1974.) DM 98.30; \$40.10.

chapters that deal with galactic subjects, from the last three, which explore extragalactic objects.

The book more than spans the gap that exists between current textbooks and current reviews in radioastronomy. (Of the former there are, because of the swift developments in all fields of astronomy, only one or two worth buying; the latter are, however, numerous, both under auspices of the International Astronomical Union and in several annual and summer school series.) There is no editorial uniformity in the treatment of first principles or the completeness with which current insights are presented. The editors seem to have limited their rule to the choice and sequence of chapters and authors, and thus achieve coherence without blurring their authors' diversity. Some of the chapters are authoritative reviews,

others unpretentious sketches, and a few are highly personal accounts. All are worth reading, some deserve and require close study. Many chapters, singly or in groups, provide an admirable means of progressing soundly from basic matters to current journal literature; thus they attain a major purpose of all postgraduate courses. Active research astronomers found time to write this excellent book and aspiring researchers will spend their time well in reading from it, to each his own selection.

No completeness is possible on this vast plain of astronomy. Of the omissions only the topic of continuum emission from normal galaxies and their nuclei is regrettable, all the more so since the present director of this same NRAO did the pioneering work. But I regard this book as a fitting closure of radio-astronomy's adolescence. Well nigh every astronomical object and astrophysical process is now touched by this technique and this scope makes it imperative to deal with themes and subjects in a multi-spectral context; this is the case even when discussing objects that are first and foremost radio sources.

Symposia or books confined to radio astronomy will increasingly combine subjects that lack all affinity and separate those which are naturally all of one piece. To acknowledge this means to adapt courses of instruction, conferences and even the structure of organisations such as the International Astronomical Union; it is the recognition that radioastronomy has come of age. □

Now that Magnus Pyke has become a television personality, known to the public for his extraordinary antics which somehow succeed in explaining scientific principles, a new book bearing his name might be expected to use the same type of technique. Those who buy *Success in Nutrition* expecting a good laugh combined with a little painless and easily assimilated knowledge are likely to be disappointed. This book, although well-written and easy to read, is a straightforward and orthodox textbook, aimed at 'O' level and 'A' level students of food and nutrition, 'A' level students in home economics and those taking OND and HND courses in catering and

hotel management. It is also recommended for the serious reader following a self-study course. As a

Pyke's guide to nutrition

Success in Nutrition. By Magnus Pyke. Pp. x+227. (John Murray: London, 1975.) £1.95.

textbook it is excellent. All the various constituents of our diet are described, details being given of their occurrence in different foods,

and the effects of cooking and preparing them on the different ingredients. Dr Pyke is always careful to point out gaps in our knowledge, and to ridicule the absurd statements produced by the faddist and the crank. Some readers may think that he is overgenerous in his commendation of the food-processing industry. The only gap in the text is the omission of any statement about 'roughage'. There seems little doubt that civilised people may suffer from the lack of fibre in their food, and that its replacement relieves constipation and many more serious complaints. I would have been interested in Dr Pyke's comments. **Kenneth Mellanby**

Particles in the sea

Suspended Solids in Water. (Marine Sciences, vol. 4.) Edited by Ronald J. Gibbs. Pp. xiii + 320. (Plenum: New York and London, 1974.) \$34.90.

THE pioneering observations by N. G. Jerlov of the light-scattering properties of sea water, obtained during the Swedish Deep-Sea Expedition (1947–48), established their usefulness for delineating the distribution and relative concentrations of suspended particles in the sea. Interest in this topic has been revived fairly recently by advances in several disciplines, including those concerned with the influence of dilute particle suspensions on the stability and mechanism of fine-grained sediment motion, with the widespread occurrence of high concentrations of particles close to the deep-sea floor (the so-called nepheloid layer) and with developments in marine optics, which have produced some novel designs of *in situ* light-scattering and transmission meters. This volume represents the proceedings of a symposium of the same title, held in Santa Barbara, California, during March 1973, at which recent work on these topics was intensively reviewed.

The first section of the book contains an introductory chapter by the editor and a chapter describing a theoretical study of the settling and reaction of particles in natural waters. In Section II, five chapters deal with the requirements of optical instruments for *in situ* use and the problems of measuring certain optical parameters of sea water. This section is a useful summary of the state-of-the-art.

Sections III and IV, occupying the major part of the book, contain case studies of suspended matter in nearshore and offshore environments using, in most cases, a combination of *in situ* light-scattering data and some measure of the total quantity of suspended material in discrete water samples. The interest here has been to use the optical data as a convenient measure of the concentration of suspended particles and then to examine the role of such material in the redistribution and sedimentation of organic and inorganic solids in the sea.

A particularly good example of an integrated approach to this type of problem is provided by Biscaye and Eitrem who describe a time series of observations of the nepheloid layer at two stations in the western North Atlantic, using measurements of excess radon to study the effect of vertical mixing on the distribution of bottom particles.

The last two chapters show that it is possible to identify sources of suspended particles from a knowledge of their chemical and mineralogical composition. This type of information will be required in studies of the generation and dissipation of nepheloid layers and the role these layers might have in the overall

sedimentary economy of the deep sea. What seems to be required is a calibration of these techniques by determining the major and minor element composition of suspended material over an entire water column, and not simply the near-bottom layers in isolation. Such information will, I hope, be made available by the GEOSECS programme of the US International Decade of Ocean Exploration.

S. E. Calvert

Behaviour differences

Sex Differences in Behavior. (Seminars in Human Reproduction.) Edited by Richard C. Friedman, Ralph M. Richart and Raymond L. Vande Wiele. Pp. xvi + 495. (Wiley: New York and London, December 1974.) £12.50.

THIS book contains 24 chapters by over 30 different authors, based on a conference held in New York in September 1973. The common theme is behavioural sex differences, but many of the chapters have little else in common. A wide variety of different approaches within psychology and biology are represented, so that the subject is considered from physiological, clinical, evolutionary, psychoanalytical, cognitive developmental, ethological, and experimental psychological viewpoints. Such diversity has resulted in a very uneven book, and this is further intensified by there being a mixture of research papers, surveys of an author's own research, and more general reviews.

The most useful chapters are those which present a survey of an author's own research, particularly where this covers several years' work. Such chapters include those by Moss, on human mother–infant interactions, Phoenix, on prenatal hormones and primate behaviour, Ehrhardt and Baker, on foetal androgens and human sex differences, Sackett, on sex differences in responses to maternal deprivation, Rosenblum, on mother–infant attachment in primates, and Green, on boys who preferred a feminine role. All of those chapters will be useful to research workers, and possibly to advanced undergraduates. Other chapters of that type—for example, that by Lewis and Weinraub on sex differences in infant attachment—are marred by the excessive detail the authors present in reporting their own data.

Chapters consisting of critical but selective reviews are also very useful in a book of this sort, and so are reviews which attempt to organise and integrate previous findings in a new way. I found the reviews by Meyer-Bahlberg, on the XYY syndrome, Levine, on sex differences in rats' responses to neonatal stress, Korner, on human neonatal differences, and Whalen, on problems and concepts in research on sexual differentiation, the most useful. Two other reviews, by Moyer, on sex differences in aggression,

and Hamburg, on an evolutionary perspective on behavioural sex differences, both conspicuously lacked critical appraisal of their material. In addition, the former merely presented material which will be published elsewhere.

Several other chapters give incomplete accounts of research work in progress, sometimes based on very small samples, and thus meriting at best only a short report in a journal. For example, Michael *et al.* base a chapter on the bisexual behaviour of female monkeys simply on observations from two animals; Kohlberg and Ullian, on the development of gender concepts, report data from two subjects (out of 70 studied); Meyer-Bahlberg *et al.*, on cryptorchidism and gender identity, use results from 10 subjects (out of 67 studied). Perksy's chapter on the menstrual cycle and psychological test measures also uses partly analysed data. The only really successful chapter reporting original experimental work is that of Doering *et al.*, on testosterone and psychological test measures in men, and even that is really more appropriate for a journal.

Overall, the book is very diverse and very uneven, so that at £12.50 it is unlikely to attract many buyers. Parts of it will be useful to a wide variety of people for a wide variety of reasons, and I would anticipate that there will be a heavy demand for library copies. John Archer

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